Altered Ca^{2+} signaling in enamelopathies[*]

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Abstract

Biomineralization requires the controlled movement of ions across cell barriers to reach the sites of crystal growth. Mineral precipitation occurs in aqueous phases as fluids become supersaturated with specific ionic compositions. In the biological world, biomineralization is dominated by the presence of calcium (Ca^{2+}) in crystal lattices. Ca^{2+} channels are intrinsic modulators of this process, facilitating the availability of Ca^{2+} within cells in a tightly regulated manner in time and space. Unequivocally, the most mineralized tissue produced by vertebrates, past and present, is dental enamel. With some of the longest carbonated hydroxyapatite (Hap) crystals known, dental enamel formation is fully coordinated by specialized epithelial cells of ectodermal origin known as ameloblasts. These cells form enamel in two main developmental stages: *a)* secretory; and *b)* maturation. The secretory stage is marked by volumetric growth of the tissue with limited mineralization, and the opposite is found in the maturation stage, as enamel crystals expand in width concomitant with increased ion transport. Disruptions in the formation and/or mineralization stages result, in most cases, in permanent alterations in the crystal assembly. This introduces weaknesses in the material properties affecting enamel's hardness and durability, thus limiting its efficacy as a biting, chewing tool and increasing the possibility of pathology. Here, we briefly review enamel development and discuss key properties of ameloblasts and their Ca^{2+}-handling machinery, and how alterations in this toolkit result in enamelopathies.

Keywords

1. Introduction

The evolutionary history of most mammals and their ancestors can be garnered from the analysis of their fossilized dental remains. Tooth size, shape, and enamel microstructure

tracks evolution in detail because dental enamel, the outer covering of teeth, is the hardest and most mineralized tissue of vertebrates, and can withstand the trials of time reasonably unchanged. This is not to suggest that enamel formation is a passive event, but the contrary. Dental enamel formation is a remarkable example of cell-tissue interactions encompassing a number of transformations in cytoskeletal elements concomitant with functional differences [1,2]. Ameloblasts are ectoderm-derived epithelial cells responsible for orchestrating the formation and mineralization of dental enamel. Their organized assembly of enamel matrix proteins and interaction with the crystal phase occur extracellularly with limited control by the enamel cells [3]. The geometry of the crystals is guided by the assembly of proteins, many of which are unique to the ameloblasts' protein synthesis machinery [4]. The mineral phase that constitutes the fully mineralized tooth enamel is composed of calcium Hydroxyapatite (Hap) organized into some of the largest known Hap crystallites in the biological world [3] (Fig. 1).

The biogenesis of enamel (amelogenesis) is, for simplicity, commonly reduced to two main developmental stages: *a)* secretory; and *b)* maturation. Secretory ameloblasts develop a distal secretory process and initiate enamel matrix protein secretion (see below) moving in predetermined outward paths toward the outer enamel [5]. Developmentally, the earliest formed regions of the enamel are the cusps, i.e. the pointy surfaces of a chewing (molar) tooth, and the last developmental phase of enamel occurs at the base. Thus, enamel forms outward and downward. Secretory ameloblasts will transition into a maturation stage cell and, rather than dedicate themselves to synthesizing and secreting enamel matrix proteins, they engage primarily in ion transport. Enamel crystal growth magnifies at this time [6]. It is during the maturation stage that many Ca^{2+} handling proteins and Ca^{2+}-associated functions are upregulated [2] in line with the increased mineral uptake and crystal growth [7]. Ca^{2+} is a key and necessary component of enamel. Therefore, disruptions in Ca^{2+} transport may cause the enamel to mineralize poorly, or improperly, resulting in enamelopathies. Here, we review the effects of altered calcium homeostasis on enamel disease. To better understand these effects, we first provide an overview of the ultrastructure of enamel cells, emphasizing the characteristics of Ca^{2+} handling organelles in these cells.

2. Enamel organ and matrix proteins

Secretory ameloblasts are post-mitotic, tall columnar, and heavily polarized cells. The cell skeleton may extend ~70 μm in height (5 μm diameter), with the nucleus found at the basal pole (away from the enamel). Mitochondria densely occupy the narrow infranuclear cytoplasm (Fig. 2A & 2B), whereas extensive endoplasmic reticulum (ER) networks populate the large infranuclear space. Abundant Golgi saccules are also present [8]. One of the most conspicuous features of secretory ameloblasts is the presence of an apical (facing the enamel) cellular extension known as the Tomes' process. Many cellular bodies fuse to the Tomes' process to exocytose and endocytose proteins (Fig. 2B). Cell-cell connections (desmosomes, tight junctions) are found both apically and distally, maintaining narrow intercellular spaces [8]. This limits the movement of molecules between cells in any direction [6]. The protein synthesis apparatus is committed to the production of a key enamel-specific protein known as amelogenin, which constitutes about 90% of all secreted products [9]. Amelogenin is required for the ordering, or alignment, of enamel crystallites

in the extracellular space [3]. Other "non-amelogenin" products including enamelin and ameloblastin, are also involved in the normal development of enamel [10]. Mutations in these enamel-specific genes result in a broad range of clinical pathologies known as *amelogenesis imperfecta* (AI), defined by abnormal mineralization or deficient growth of the enamel layer [11].

Many secretory cells will transition into a maturation stage ameloblast, showing reduced height but maintaining their polarity, and losing the secretory Tomes' process. They develop deep membrane infoldings or ruffled distal border, which plays an important role in ion transport [6] (Fig. 2C & 2D). These ruffled ameloblasts have less conspicuous ER tubules and contain mitochondria both at the infra- and supranuclear regions with large clusters near the ruffled end [12] (Fig. 2C, Fig. 3). Distal cell-cell tight junctions (TJ) constrain the movement of molecules and ions between cells toward the enamel [6]. Unusually in mammalian cell biology, these ruffled cells will lose and reform these distal infoldings, periodically adopting a smooth distal pole [13] (Fig. 3). These smooth-ended cells, which are a minority of all maturation stage cells, show increased intercellular spaces and distally placed mitochondria. There is only limited knowledge of their role in amelogenesis. It has been suggested that the smooth ameloblast phase facilitates the diffusion of organics from the enamel into the interstitial fluid. They have also been associated with stabilizing pH in the enamel compartment from the mildly acidic pH observed during the ruffled-ended stage [6,14]. An important feature of all maturation stage ameloblasts is their connection with adjacent (basally) cells of the papillary layer (PL). The PL cells contain many mitochondria and likely form a functional syncytium with the ameloblasts [15] (Fig. 2C, Fig. 3). Maturation stage cells orchestrate the bulk of ion transport necessary for the massive expansion of enamel crystals [1,6,15].

Given the presence of many junctional complexes communicating adjacent secretory as well as maturation stage ameloblasts, the general consensus is that ameloblasts are the active modulators of transepithelial transport. They do so in a highly organized fashion, with little contribution from paracellular transport [16–18]. This notion is reinforced by the presence of a basal membrane serving as anchorage between maturation ameloblasts and the crystals, adding to the limitations in the passage of interstitial fluid to the enamel (Fig. 3) [19]. It is also intuitive to suggest that the highly structured nature of the biomineralization process of enamel necessitates a delicate balance controlling ion fluxes to coordinate their incorporation into the forming crystals to regulate its stoichiometry. Thus, any alterations that occur either in the secretory (protein synthesis) or maturation (mineralization) stages can upset the delicate balance of enamel crystal assembly.

3. General effects of systemic calcium changes in enamel

Ca^{2+} is the fifth most abundant element in the human body weighing about 1000 g, most of which (99%) is found in bone as Hap [20]. Bone Ca^{2+} serves not only as a stiffening material in the skeleton, but also as the main Ca^{2+} reservoir in the body. Ca^{2+} metabolism is mediated by renal reabsorption, intestinal absorption, and bone turnover which are dynamically regulated by parathyroid hormone (PTH), 1,25-dihydroxyvitamin D [1,25(OH)2D], and Ca^{2+} itself [20]. Bone tissue is a dynamic system that is constantly being

remodeled (bone deposition and bone resorption) so that short-term Ca^{2+} deficiencies can be resolved over time. Ca^{2+} is released from bone by the activity of PTH, causing its levels to rise in plasma where it is closely monitored, being removed by calcitonin stimulation when levels are too high and then stored in bone. Although enamel is more highly mineralized than bone containing increased levels of Ca^{2+}, this trapped Ca^{2+} within the enamel crystal lattice cannot be "released" dynamically into the system as it does in bone since enamel does not remodel. However, there has been interest in determining whether disruptions in systemic calcium levels (hypo-hypercalcemia), via PTH or vitamin D (and its receptor VDR), have lasting effects in enamel.

Bonucci and co-workers reported the effects of hypocalcemia in young rats induced by low dietary Ca^{2+} in nursing mothers [21]. This resulted in hypomineralized enamel in the pups. Researchers also found that this defect could be corrected following a 10-day period of added Ca^{2+} in the diet. Hypocalcemia was also reported to affect the enamel cells, resulting in the formation of cyst-like structures in the ameloblasts layer and abnormal localization of enamel proteins [22]. In a surgical study, thyroidectomy did not affect enamel in rats but hypocalcemia induced by thyro-parathyroidectomy resulted in severely affected enamel [23]. These data link hypocalcemia with enamel deficiencies, as expected.

In addition, the role of vitamin D has been recognized in enamel [24] among other reasons, as it appears to regulate enamel gene expression [25]. The role of vitamin D in enamel is also supported by reports that deficiency of vitamin D during pregnancy results in increased early childhood caries [26]. This is validated by a study that reported disruptions in the formation of enamel as well as disrupted calbindin expression in vitamin D deficient rats [27]. *VDR*$^{-/-}$ mice also showed enamel abnormalities although the effects of loss of function of VDR were complex in the context of induced dietary hypocalcemia or in its absence [25]. Other studies analyzed the enamel phenotype of Rickets patients, a condition associated with vitamin D-deficiency, identifying increased caries and tooth decay in this group [25]. However, other vitamin D-deficiency related diseases, including Alzheimer's disease (AD) and Parkinson's disease, have not been associated with enamel abnormalities to date. The recently proposed role of vitamin D by Berridge [28] as a regulator of intracellular signaling pathways and redox may be of interest in the future of enamel biology as Ca^{2+} signaling and redox appear to be tightly associated with enamel mineralization.

Additional effects in enamel linked to systemic dysregulation in Ca^{2+} homeostasis were recently highlighted in patients with nephrocalcinosis [29]. This disease results from an increase in the Ca^{2+} content of the kidneys due to increased urinary levels of calcium, phosphate, and oxalate, and leads to progressive renal damage. Nephrocalcinosis patients were reported to show AI and identified mutations in the *FAM20A* gene associated with this phenotype [30]. *FAM20A* is a kinase expressed in secretory stage ameloblasts and loss of function mutations prevent phosphorylation of proteins in the secretory pathway causing pathology [30].

4. Channels involved in Ca^{2+} dysregulation in enamel

As stated above, the transcellular movement of Ca^{2+} is favored over a paracellular route [16], a model that would thus require Ca^{2+} channels, pumps, and exchangers as mediators of Ca^{2+} uptake and extrusion [17].

4.1. Store-operated Ca^{2+} entry

The bulk of Ca^{2+} uptake in enamel cells is regulated by store-operated Ca^{2+} entry (SOCE) channel [16,31]. The SOCE archetype is the Ca^{2+} release activated Ca^{2+} (CRAC) channel formed by two protein families, the ER-based single pass membrane protein STIM1 (stromal interaction molecule) and its homologue STIM2, as well as the plasma membrane bound Orai proteins [32–34]. STIM1 and STIM2 are Ca^{2+} sensors and have a luminal EF hand Ca^{2+} binding domain. Upon a reduction of ER Ca^{2+} concentration and its detachment from the EF hand, STIM1 proteins undergo conformational changes leading to physical interactions with the Orai proteins, allowing sustained Ca^{2+} entry [35,36]. There are three mammalian Orai proteins, Orai1–3, all of which function as membrane channel pores [37,38]. Despite the similar roles of STIM1 and STIM2 as Ca^{2+} sensors and of all three Orai proteins as functional membrane pores, only mutations in *STIM1* and *ORAI1* genes are known to cause disease [39]. These so called CRAC channelopathies include an ensemble of dysfunctions, the chief being immunodeficiency among the loss-of-function mutations, and result in both AI and a form of ectodermal dysplasia [39,40]. In support of the role of CRAC channels in enamel formation, pharmacological approaches using a number of Orai inhibitors (Synta-66, BTP-2, 2-APB) have demonstrated a significant reduction in Ca^{2+} influx in thapsigargin stimulated cells [31]. More recently, a double conditional deletion of *Stim1* and *Stim2* genes in mice reported severe enamel hypomineralization in this mouse model with a gross dental phenotype resembling human AI phenotype [41].

4.2. $Ca_v1.2$ channels

The L-type voltage-gated Ca_v1 ($Ca_v1.2$) channel encodes an alpha-1 subunit of a voltage-dependent calcium channel. Loss-of-function mutations in the encoding gene (*CACNA1C*) result in Brugada syndrome, producing short QT intervals potentially resulting in sudden cardiac death [42]. Gain-of-function mutations in $Ca_v1.2$ result in sustained inward Ca^{2+} currents due to nearly complete loss of voltage-dependent channel inactivation and intracellular Ca^{2+} overload in multiple cell types, causing a multiorgan dysfunction known as Timothy syndrome [42]. Timothy syndrome patients present with dental abnormalities, including smaller teeth and abnormal enamel [43], likely due to dysregulation of the membrane potential of the enamel cells and the resulting Ca^{2+} overload, but this has not been studied to date. Yet, it is noteworthy that in vascular smooth muscle cells, STIM1 interacts with $Ca_v1.2$ at localized ER/plasma membrane junctions that also contain Orai1 channels, thus controlling $Ca_v1.2$ Ca^{2+} signals [44]. This interaction is not known in enamel cells.

4.3. TRP channels

Transient receptor potential (TRP) channels have recently come to light in association with enamel. The nearly ubiquitous, melastatin-like transient receptor potential cation channel

(TRPM7) is an essential permeable ion channel regulating Mg^{2+} homeostasis [45]. While homozygous deletion of the kinase domain in the c-terminus of TRPM7 is embryonic lethal [45], heterozygous mice are viable for the study of TRPM7 in enamel. These mice showed severely hypomineralized enamel and skeletal abnormalities with lower Ca^{2+} and higher Mg^{2+} content in the enamel [46]. Interestingly, recent evidence by Penner and colleagues has linked TRMP7 with SOCE, and while TRPM7 itself is not a store-operated ion channel, its activity via the kinase domain modulates STIM1, thereby participating in Ca^{2+} homeostasis [47].

5. Ca^{2+} buffers and pumps

Intracellular Ca^{2+} buffers have been reported in enamel cells including parvalbumin, calretinin, calmodulin, and calcineurin [reviewed in [16,48]] but, to date, no enamel deficiencies have been associated with mutations in any of these genes. In addition, ATP-dependent Ca^{2+} pumps, including sarco/endoplasmic reticulum Ca^{2+} ATP-ases (SERCA) and plasma membrane Ca^{2+} ATP-ases (PMCA), have been recently revisited as the role of Ca^{2+} in both signaling and transport in enamel has been under scrutiny [16,49]. Mutations in SERCA2, the predominant isoform in enamel cells [50,51], have not been associated with enamel disease. PMCA1 and PMCA4 are the predominant isoforms found in enamel cells notably showing a basolateral localization [49]. These pumps were originally considered to play a major role in Ca^{2+} transport in enamel [52], but no mutations have been identified in the PMCA gene coding family (*ATP2B1–4*) that would result in enamel abnormalities. Thus, they may instead play a minor role in maintenance of Ca^{2+} homeostasis without being essential components.

6. Calcium exchangers

Both NCX and NCKX are expressed in enamel cells. These bidirectional electrogenic transporters move Na^+ in exchange for Ca^{2+} (and K^+ in the case of NCKX). The NCX family (encoded by *SLC8A* gene) contains six members, but only NCX1 and NCX3 are clearly expressed in enamel cells as showing an apical or apico/lateral distribution [53]. Electrophysiological studies of whole cell recordings of primary enamel cells as well as of the use of pharmacological inhibitors supported the functionality of NCXs in enamel cells [53]. However, no dental abnormalities reported to date have been associated with *SLC8A* gene mutations.

NCKX4 is a sodium/potassium/calcium exchanger (encoded by *SLC24A4*) that operates at a $4Na^+$: $1Ca^{2+}$ +1 K^+ stoichiometry [54]. NCKX4 was first identified in rat enamel cells by a genome-wide screening identifying *Slc24a4* transcript as highly upregulated in maturation stage enamel cells [2]. Immunohistochemistry showed absence of NCKX4 in secretory cells, but demonstrated a strong apical localization in maturation stage ameloblasts suggesting a direct role in Ca^{2+} extrusion in these cells [55]. Subsequent studies supported this role for NCKX4 with the identification of hypomaturation and AI in consanguineous patients with null and missense mutations in the *SLC24A4* gene [56]. *Slc24a4*$^{-/-}$ mice also presented with abnormal enamel reminiscent of the human AI phenotype [56]. Later studies identified a novel homozygous [57] and a missense mutation in *SLC24A4* causing hypomaturation

and AI [58]. In patients with *SLC24A4* mutations, AI is manifested as loss of enamel, increased attrition, and heavy-brownish discoloration, all indicative of hypomineralization. More recently, the sequencing of candidate genes in a consanguineous family presenting with AI similar to that described previously (22, 23 [58]) identified full and partial deletions in the last three exons of the *SLC24A4* gene caused by homologous recombination [59]. Besides the human and murine genetic studies, Samoyed dogs, the large herding-sized dog of the Samoyedic people of Siberia, were also reported to have a mutation in the *SCL24A4* gene associated with AI [60]. These genetic data strongly support the role of NCKX4 in Ca^{2+} homeostasis in enamel cells and point to its natural role as a key mediator in Ca^{2+} extrusion.

More recently, a functional assessment of *SCL24A4* mutations associated with AI and their impact on cytosolic Ca^{2+} levels were performed in HEK293 cells in the reverse operating mode of NCKX4 [61]. Mutant HEK293 cells showed decreased cytosolic Ca^{2+} (measured with Fluo-4FF), further strengthening the role of NCKX4 in Ca^{2+} transport in enamel [61]. Its role in enamel appears to be impacted by its localization in the ruffled-border. In conditions where Ca^{2+} homeostasis is disrupted [41] or in situations of other physiological insults such as fluorosis [62], NCKX4 localization is altered with strong signals found in the cytosol. This alteration is associated, in at least one instance, with the loss of the ruffled-border [41]. This suggests that this apical membrane feature is necessary for NCKX4 to function as a Ca^{2+} exchanger and to enable Ca^{2+} extrusion. This is supported by the loss of NCKX4 expression in smooth-ended ameloblasts [16].

In addition to its now accepted role in AI, NCKX4 has been linked to olfactory responses [63], melanocortin-dependent satiety [64], and, most recently, to vision, with NCKX4 shaping the cone photoresponse [65].

7.   The role of intracellular organelles

Several studies have defined the cellular localization of ER tubules and mitochondria in enamel cells at each developmental stage [reviewed in [15]] although only limited knowledge of their putative functions (e.g. their contribution to Ca^{2+} signaling) in enamel formation is available. The expression of inositol 1,4,5-triphosphate receptors (IP$_3$R) in enamel cells was originally described by Hubbard [66] and later by our group, identifying the expression of all three IP$_3$R isoforms [31]. Ryanodine receptors (RyR) were also reported in ameloblasts, but their expression was much less than that of IP$_3$R [31]. Our ongoing investigation of the effects of physiological agonists in enamel cells (unpublished) lends support to the dominant role of IP$_3$R, rather than RyR, in enamel. Despite the relevance of ER Ca^{2+} signaling in cell biology, to date there are no known dental defects associated with mutations in IP$_3$R. Yet, there is growing interest in enamel biology by a number of laboratories, including our own, on one of the key ER functions, the unfolded protein response (UPR) [41,67–69]. UPR has been linked to disruptions in enamel formation, but the mechanism affecting enamel is different. Two recent reports have described mutations in *AMEL* and *ENAM* that appear to induce UPR. The murine p.Tyr64His mutation in the *AMEL* gene caused UPR-induced apoptosis resulting in AI [69], whereas the ENAMEMp.L31R mutation resulted in UPR and disruptions in the secretory

pathway of ameloblasts, ultimately leading to AI [67]. Our work showed that UPR was linked to dysregulation of Ca^{2+} homeostasis but did not have a clear effect on the expression of enamel genes [41].

Mitochondrial Ca^{2+} dynamics and the role of mitochondrial-associated functions such as cell metabolism and cell redox are all poorly known in enamel cells. To date, there are no known mitochondrial genes that are recognized as impacting enamel formation when mutated. This is likely to be a misrepresentation, however, given that alterations in enamel are not always severe or obvious to dental practitioners and primary physicians. In fact, as shown in Fig. 4, enamel cells show high Ca^{2+} buffering capacity by mitochondria, suggesting a role in Ca^{2+} signaling.

Peroxisomes are also known to participate in Ca^{2+} homeostasis in cells [70]. Although their role in enamel is limited, recent data have shed light on the expression of a number of proteins associated with peroxisomal function during the early stages of amelogenesis [71]. It is also reported that patients with abnormal peroxisome biogenesis present with facial dysmorphology and abnormal enamel [71]. More recently, mild peroxisomal dysfunction caused by hypomorphic mutations in the *PEX1* and *PEX6* genes caused Heimler syndrome – characterized by sensorineural hearing loss and nail dysplasia, and resulted in AI [72]. These data suggest that maintenance of peroxisomal function is important in enamel development although it is unclear whether a direct role in peroxisomal-mediated Ca^{2+} signaling can be established.

8. Stim1/2-deficient ameloblasts

Stim1/2-deficient mice were recently developed in collaboration with the laboratory of Stefan Feske, as reported [73], allowing us to investigate the effects of Ca^{2+} dysregulation in cells and in the enamel. The enamel of these mice were severely hypomineralized, but there were also a number of striking changes in the enamel-forming cells [41]. The ameloblasts of Stim1/2-deficient mice, for example, showed a high number of multinucleated and/or short-bodied cells. Multinucleation has been documented in wild type cells as an intriguing feature of ameloblasts observed in a minority of both secretory and maturation stage cells [74]. The interest in this feature resides in the notion that all ameloblasts are considered to be post-mitotic cells and, thus, this should not be expected. Yet, as cells age, the number of multinucleate ameloblasts increases, a process that has been linked to the fusion of two or, in some cases, more mononucleate cells [74]. While multinucleate ameloblasts are uncommon, our analysis of ameloblasts of Stim1/2-deficient mice showed higher numbers of multinucleated/shorter cells (Fig. 5B & 5C). The reasons for these alterations in the ameloblasts of these mice are unknown, but disrupted Ca^{2+} homeostasis likely played a role.

Closer inspection of the ameloblasts of Stim1/2-deficient mice by transmission electron microscopy (TEM) revealed that maturation ameloblasts did not develop a typical ruffled-border [41] (Fig. 6A). These ameloblasts also showed displaced mitochondria and in many of them the cristae had been disrupted by non-electron dense, multilayered bodies resembling mitophagic structures [41] (Fig. 6B).

Bioinformatic analysis of RNAseq data of enamel cells of these mice, however, revealed an unexpected link to the glutathione system. It was shown that the glutathione metabolism pathway was one of the most enriched in enamel organ cells [41]. We confirmed this by real-time PCR showing downregulation of glutathione S-transferases, which are important enzymes in protein S-glutathionylation [75]. Our hypothesis that the glutathione system was altered in Stim1/2-deficient mice was supported by the lack of actin expression in the distal (apical) pole of the cells but not in the wild type cells. Actin is one of the best known targets of S-glutathionylation [76] and dysregulation results in altered cell cytoskeleton [77]. Thus, we surmised that disruptions in Ca^{2+} homeostasis associated with abnormal SOCE affected the glutathione system and, in turn, impacted the ability of actin to support the formation of a ruffled-border [41].

Another known target of S-glutathionylation is the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is an ATP-dependent chloride channel that controls salt and water transport across epithelial tissues. CFTR mutations principally cause exocrine pancreatic destruction and airway disease through infection and inflammation, also producing intestinal obstruction and neuropathy [21]. This anion channel has been shown to be important in enamel mineralization despite the very low level of chloride present in the enamel crystals (~0.0065 mol/g) [78–81]. CFTR function is altered by oxidized forms of glutathione which, at the single channel level, inhibited the opening rates of individual CFTR channels, likely via a poorly conserved cysteine (cys-1344) found in a region predicted to participate in ATP-dependent channel opening [82]. The lower chloride concentration in the enamel of Stim1/2-deficient mice suggested that CFTR may have been impacted by abnormal glutathione. Fig. 6C and D show the localization of CFTR at the apical pole of wild type maturation stage ameloblasts but not in Stim1/2-deficient mice, where CFTR signals were weak and found across the cell cytosol. These data are in line with the role of S-glutathionylation in CFTR function, leading us to consider that glutathione metabolism plays an unexpected role in mediating ion transport in enamel cells. This potentially raises another issue; the subcellular localization of NCKX4 in ameloblasts of Stim1/2-deficient mice was abnormal as reported [41]. While we originally considered the possibility that its mislocalization was associated with the lack of a ruffled-border in the ameloblasts, it could also be hypothesized that NCKX4 is a target of S-glutathionylation. The 622 amino acids sequence of NCKX4 contains 11 cysteine residues, although currently there are no published data on S-glutathionylation of NCKX4. Other proteins such as STIM1, with fewer cysteines in a larger protein sequence than NCKX4, are s-glutathionylated [83].

9.   Non-Ca^{2+} enamelopathies

It should be noted that the altered function of a number of proteins involved in transepithelial transport in enamel cells also result in enamelopathies [1]. Some of these, such as sodium/bicarbonate cotransporters (NBCE1) and anion exchangers (AE2), are involved in acid-base balance [84], highlighting the role of pH balance in enamel [14]. There is, however, a growing list of transporters that affect enamel [85], including poorly known proteins like the sodium-coupled citrate NaCT. This protein modulates cellular uptake of citrate and results in AI associated with the rare autosomal Kohlschütter–Tönz syndrome

(KTZS), otherwise characterized by epilepsy and intellectual disability [86]. Studies of KTZS emphasize the disparity of systemic effects on enamel, but also reiterate how sensitive biomineralization is to a vast array of conditions.

10. Summary and outlook

Understanding the role of Ca^{2+} signaling and transport in enamelopathies presents new challenges when compared to other systems. Biomineralizing systems have two check points: *a)* the cell; and *b)* its output, i.e. the formation of Hap crystals. Alterations in cell kinetics can impact the latter dramatically, leading to a record of these defects in the enamel crystals. This record becomes permanently registered in enamel as it does not remodel. So sensitive are enamel cells that even periods of high fever or the dramatic moment of birth produce alterations in enamel formation that will remain registered in enamel for life [87]. The assembly of the crystals via interaction with matrix proteins and other ions occurs in the extracellular space and while this is beautifully controlled by the ameloblasts, once materials exit the cells there is limited direct control of the biochemical interactions that take place. However, ameloblasts are clearly the directors of the assemble and while some progress has been made to define their Ca^{2+} toolkit, it is now apparent that a panoply of intracellular Ca^{2+} signaling processes are yet to be elucidated, including mitochondrial Ca^{2+} signaling, the redox environment, and how these processes interact to form Hap crystals. Data described above highlight the relevance of Ca^{2+} signaling molecules in enamel disease. The severity of these pathologies is remarkable in that it has elucidated two main links to disease to date: *a)* Ca^{2+} uptake and Ca^{2+} extrusion are essential components of healthy enamel; whereas *b)* the intracellular Ca^{2+} machinery is seemingly less critical based on currently available data.

Acknowledgements

Abbreviations:

Hap	hydroxyapatite
SOCE	store-operated Ca^{2+} entry
RA	ruffled-ended ameloblast
SA	smooth-ended ameloblast
AI	amelogenesis imperfecta

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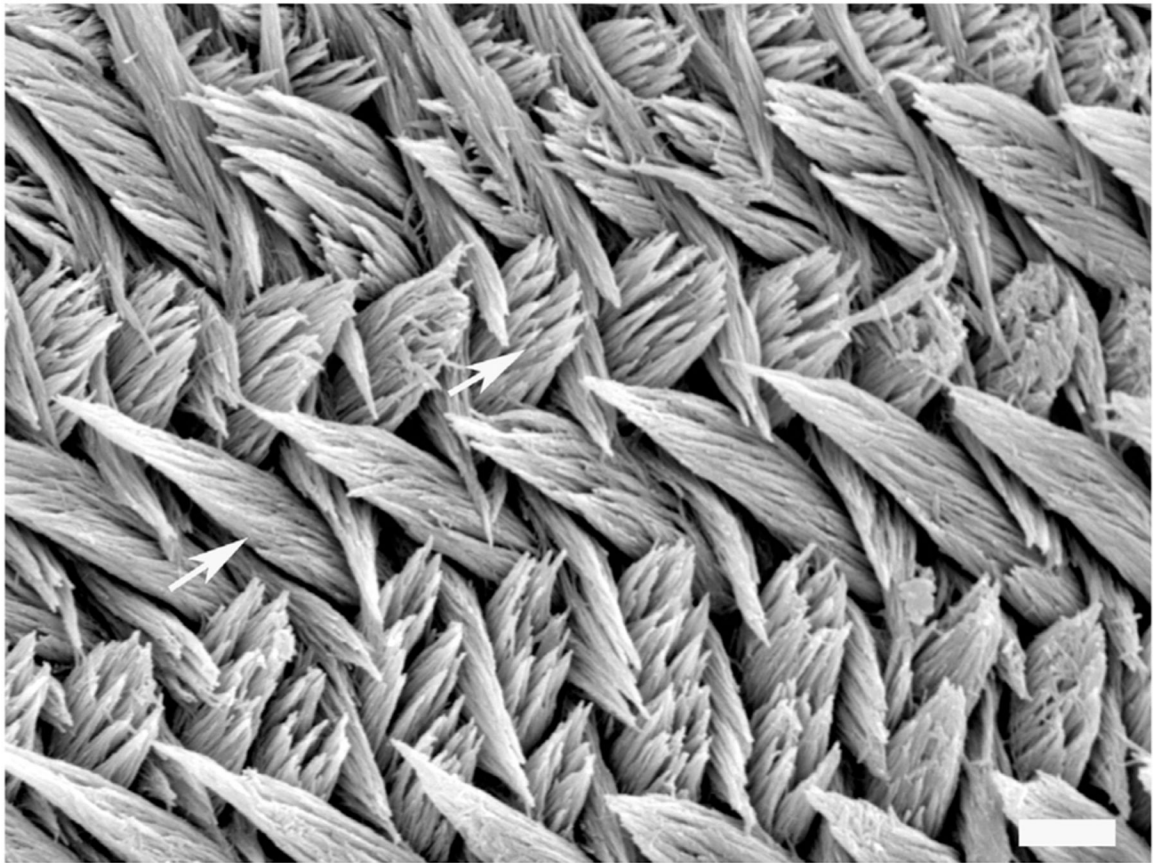
Fig. 1.
Enamel pattern in mice: Rodent enamel is commonly used as a model to study mammalian enamel development. This SEM micrograph shows enamel prisms (arrowed) oriented in different directions, which is characteristic of rodent enamel but less so in human enamel. Within the prisms, single crystals can be identified. Scale bar = 5 µm.
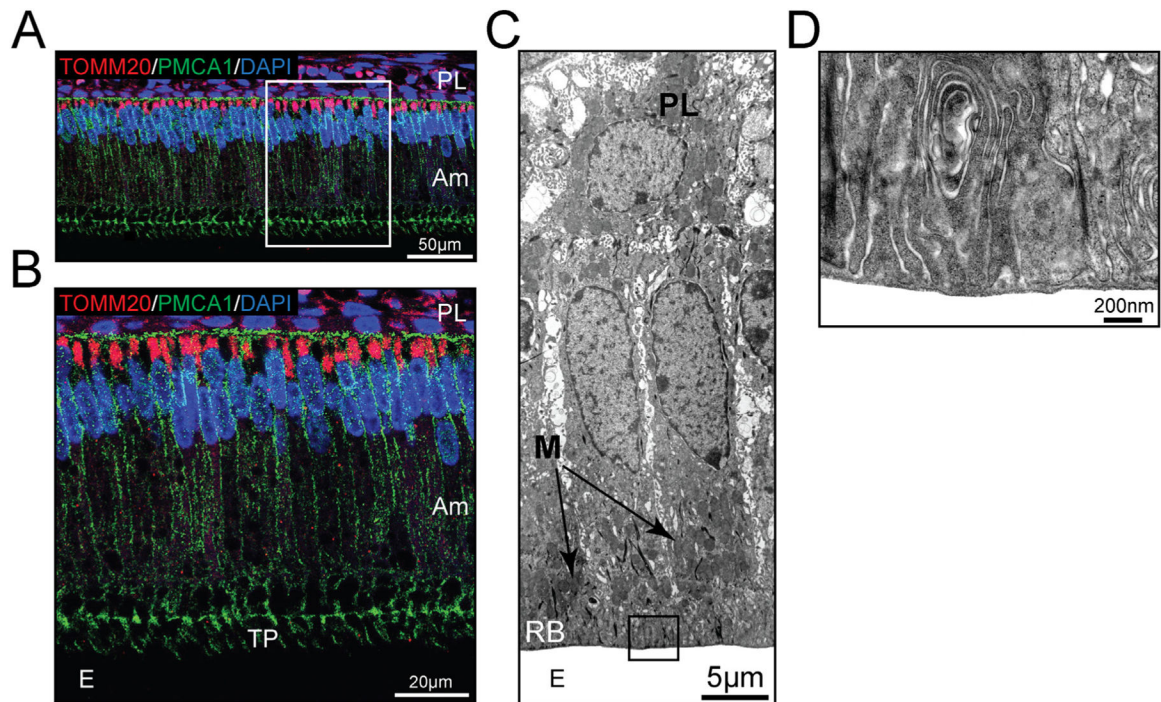
Fig. 2.
Mitochondria localization in secretory and maturation stage ameloblasts. (**A**) The
mitochondrial outer membrane marker TOMM20 (red) was used to visualize the dominant
infranuclear localization of mitochondria in secretory stage ameloblasts. The plasma
membrane of ameloblast cells was highlighted by immunofluorescence staining of PMCA
(green). Cell nuclei are stained with DAPI (blue). A close-up is shown in (**B**), which also
highlights the Tomes' process (TP), a cell extension at the apical (distal) end near the enamel
(E). (**C**) TEM image of maturation stage ameloblasts with mitochondria (M) clustering in
areas just above the ruffled-border (RB) and below the nucleus. (**D**) A close-up of the
ruffled-border showing the deep infoldings characteristic of this apical membrane structure.
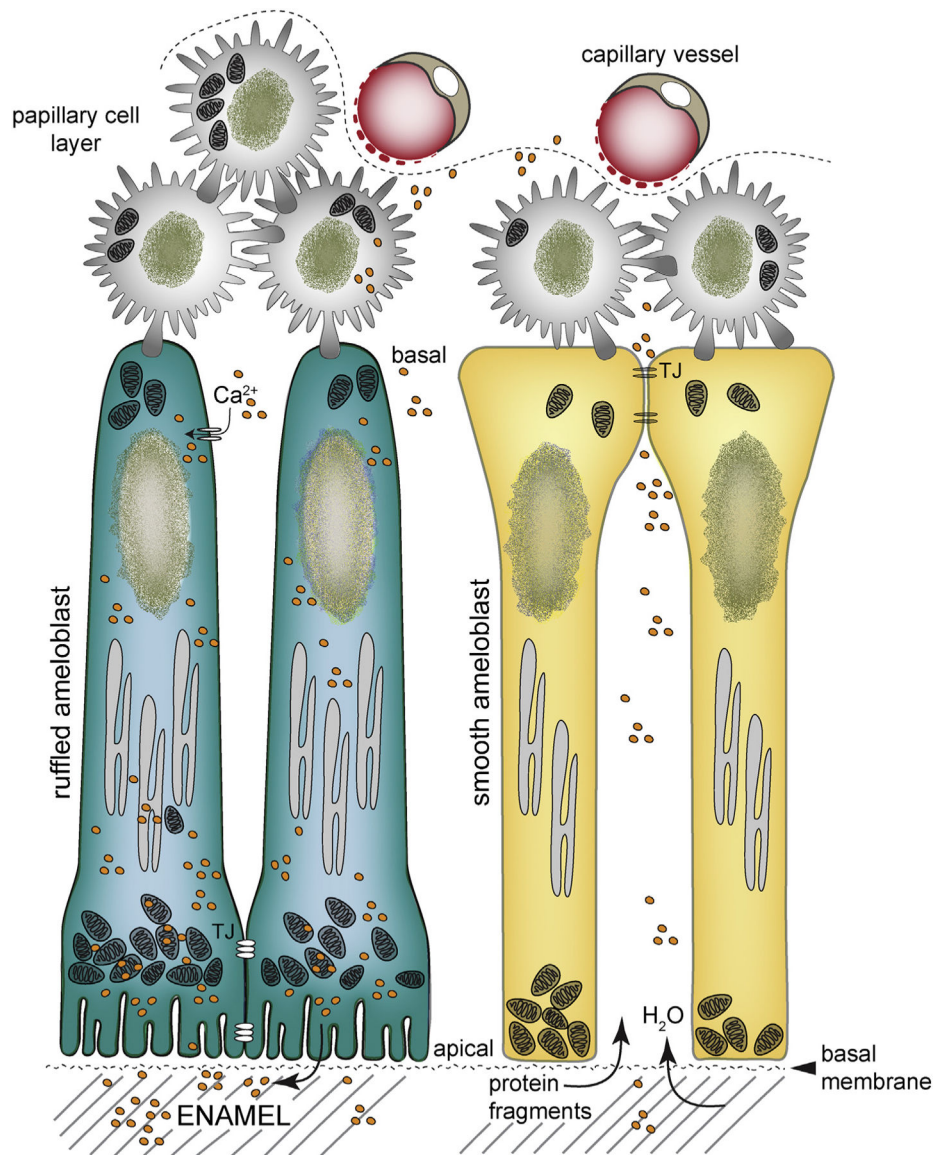Am = ameloblasts. PL = papillary cell layer.

Fig. 3.
Schematic of ruffled- and smooth-ended ameloblasts: Maturation stage ameloblasts have two main morphologies. In the ruffled-ended ameloblasts (RA), mitochondria localize to the infra- and supranuclear cytoplasm clustering between the nucleus and the ruffled-border. In smooth-ended ameloblasts (SA), mitochondria are found near the apical pole. RA have tight junctions (TJ) apically limiting the flow of ions and molecules between cells. RA is the predominant morphology (70% of cells in the maturation stage) and will cycle into SA and vice versa after a few hours several times per day. Ca^{2+} uptake is modulated by SOCE channels. Ca^{2+} ions move across the RA to reach the ruffled-border, but in the SA phase ions can move between cells. In the SA morphology, leaky TJ are found at the basal pole but do not have junctions distally. Water and protein fragments can diffuse into the wider intercellular spaces of SA. The cyclic modulation of RA to SA is poorly understood, but is required for proper enamel mineralization. Papillary cells attach at the basal pole of

ameloblasts and are in close proximity to blood vessels. A basal membrane attaches the cells to the mineralizing matrix. Modified from ref. [15].
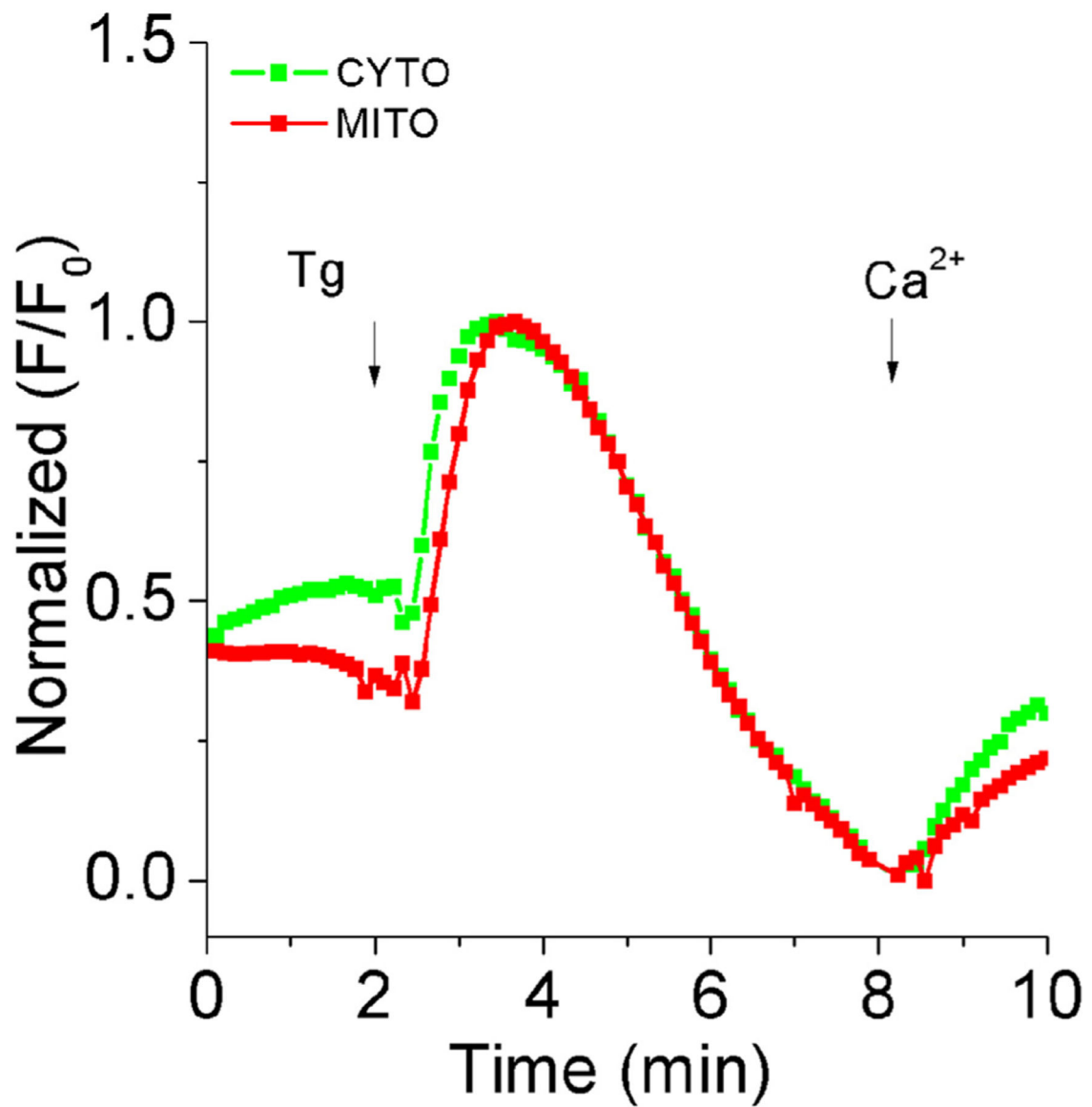
Fig. 4.
Mitochondrial and cytoplasmic Ca^{2+} recordings in the enamel cell line LS8 cells. Live-cell imaging using epifluorescence inverted microscopy of cultured LS8 cells loaded with cytoplasmic (Fura 2-AM) and mitochondrial (Rhod-2) fluorescent probes. Plot shows increase in cytosolic and mitochondrial Ca^{2+} after addition of thaspigargin (1.5 μM) in 0 Ca^{2+} and following 2mM Ca^{2+} restitution.
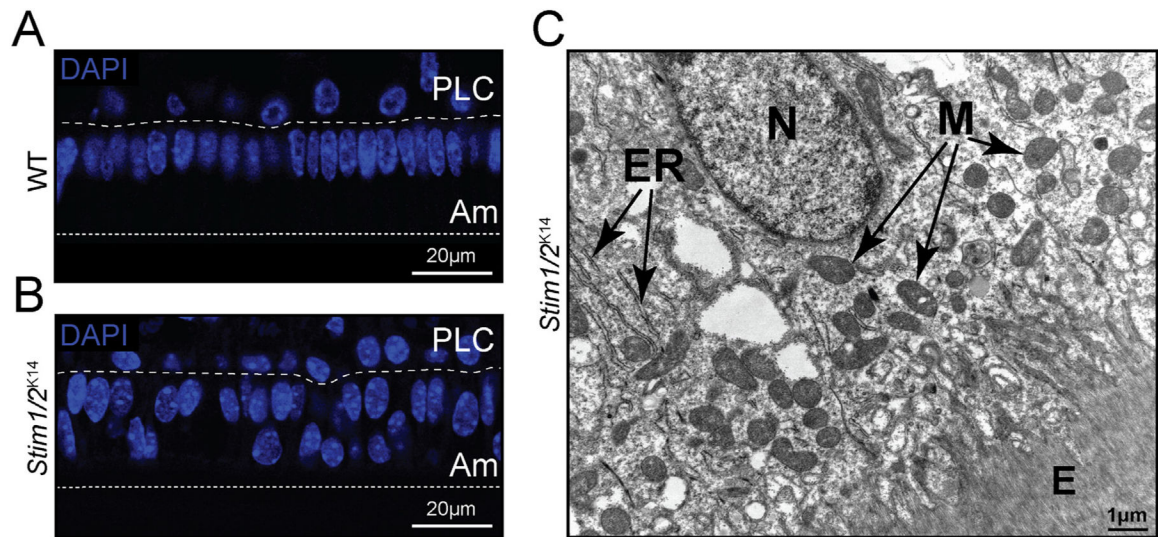
Fig. 5.
Stim1/2-deficient mice show short/multinucleated ameloblasts. (**A**–**B**) Maturation stage ameloblasts of Stim1/2-deficient mice show high numbers of multinucleated and short ameloblasts (**B**) compared to control mice (**A**) as visualized by DAPI staining (blue). (**C**) TEM image of Stim1/2-deficient maturation stage ameloblast showing a nucleus abnormally localized at the apical pole as are the mitochondria. ER = Endoplasmic reticulum. M = mitochondria. E = enamel space.
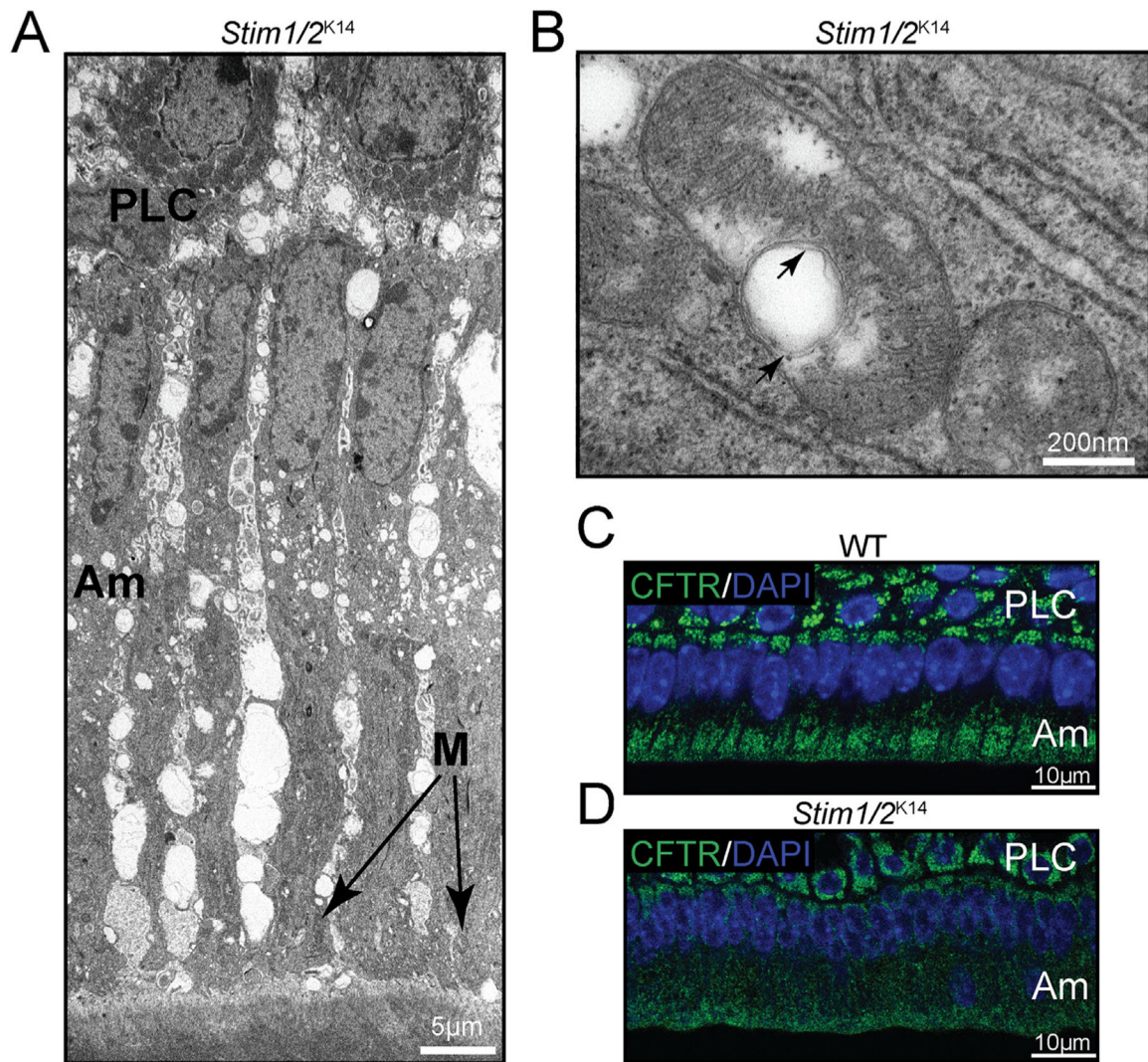
Fig. 6.
Stim1/2-deficient ameloblasts. (**A**) TEM micrograph of Stim1/2-deficient maturation stage ameloblasts (Am) showing arrested development of the ruffled-border, increased intercellular space, and apically placed mitochondria. These characteristics are atypical of ruffled-ended ameloblasts but define smooth-ended ameloblasts. Stim1/2-deficient ameloblasts analyzed in different positions of the lower incisors all showed a smooth-ended morphology, suggesting that Ca^{2+} signals are important for the formation of the ruffled-border. (**B**) Mitochondria of Stim1/2-deficient mice commonly showed white (non-electron dense) bodies with multiple outer membranes in their matrix. (**C–D**) The cystic fibrosis transmembrane conductance regulator (CFTR) protein is important in chloride transport during enamel maturation. CFTR is a key target of S-glutathionylation. In WT ameloblasts, CFTR is localized at the apical side of the ameloblasts as shown by the immunofluorescence analysis of WT ameloblasts (C), but in Stim1/2-deficient ameloblasts, CFTR is mislocalized (D), suggesting that it was not S-glutathionylated. DAPI staining (blue) was used to indicate cell nuclei.