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SYMPOSIUM REVIEW

# $Ca^{2+}$ transport and signalling in enamel cells

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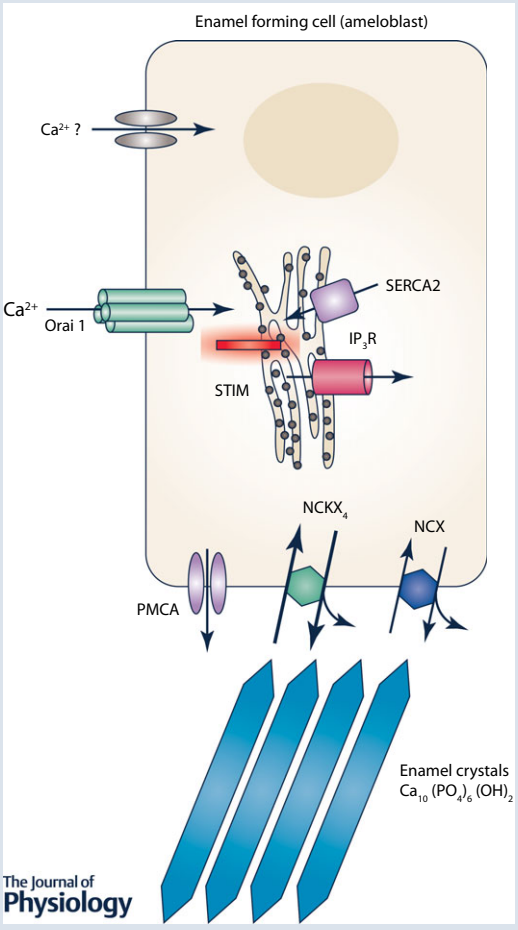


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**Abstract** Dental enamel is one of the most remarkable examples of matrix-mediated biomineralization. Enamel crystals form *de novo* in a rich extracellular environment in a stage-dependent manner producing complex microstructural patterns that are visually stunning. This process is orchestrated by specialized epithelial cells known as ameloblasts which themselves undergo striking morphological changes, switching function from a secretory role to a cell primarily engaged in ionic transport. Ameloblasts are supported by a host of cell types which combined represent the enamel organ. Fully mineralized enamel is the hardest tissue found in vertebrates owing its properties partly to the unique mixture of ionic species represented and their highly organized assembly in the crystal lattice. Among the main elements found in enamel, $Ca^{2+}$ is the most abundant ion, yet how ameloblasts modulate $Ca^{2+}$ dynamics remains poorly known. This review describes previously proposed models for passive and active $Ca^{2+}$ transport, the intracellular $Ca^{2+}$ buffering systems expressed in ameloblasts and provides an up-dated view of current models concerning $Ca^{2+}$ influx and extrusion mechanisms, where most of the recent advances have been made. We also advance a new model for $Ca^{2+}$ transport by the enamel organ.

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**Abstract figure legend** Generalized model for $Ca^{2+}$ transport in dental enamel cells (ameloblasts). $Ca^{2+}$ is transported across a semipermeable cell barrier to reach the growing enamel crystals. Recently, store-operated $Ca^{2+}$ entry has been implicated as a key $Ca^{2+}$ influx system, with inositol receptors probably involved in $Ca^{2+}$ release. SERCA2 is the most prominent refilling pump of the endoplasmic reticulum lumen. Extrusion involves the exchangers NCKX4 and two NCXs. Plasma membrane $Ca^{2+}$-ATPases (PMCAs) are also involved. The full cellular machinery used by ameloblasts to modulate $Ca^{2+}$ transport and signalling is discussed.

**Abbreviations** AMBN, ameloblastin; AMEL, amelogenin; 2-APB, 2-aminoethyldiphenyl borinate; CAD, channel activation domain; CaBP, $Ca^{2+}$ binding protein; CC, coil-coil; CCb9, CC fragment b9; CNX, calnexin; CRAC, $Ca^{2+}$ release activated $Ca^{2+}$; EMPs, enamel matrix proteins; ENAM, enamelin; ER, endoplasmic reticulum; GBHA, glyoxal bis(2-hydroxyanil); HRP, horseradish peroxidase; $IP_3$, inositol-1,4,5-trisphosphate; $IP_3Rs$, inositol-1,4,5-trisphosphate receptors; KLK4, kallikrein 4; MMP20, matrix metalloprotease 20; NCKX, $K^+$-dependent $Na^+/Ca^{2+}$ exchanger; NCX, $K^+$-independent $Na^+/Ca^{2+}$ exchanger; NFAT, nuclear factor of activated T cells; PLC, phospholipase C; PM, plasma membrane; PMCA, plasma membrane $Ca^{2+}$-ATPase; PtdIns(4,5)$P_2$ or $PIP_2$, phosphatidylinositol 4,5-bisphosphate; RA, ruffled-ended ameloblast; RyR, ryanodine receptor; SA, smooth-ended ameloblast; SOAR, STIM-ORAI activating region; SOCE, store-operated $Ca^{2+}$ entry; SAM, sterile alpha motif; SERCA, sarco/endoplasmic reticulum ATPase; SR, sarcoplasmic reticulum; STIM, stromal interaction molecule.

## Introduction

It would be intuitive to think that the role of $Ca^{2+}$ in mineralization would be best understood by examining the most highly mineralized tissue of vertebrates, dental enamel. Paradoxically, the potentially versatile role of $Ca^{2+}$ and its impact in the formation and mineralization of enamel is vastly underappreciated, remaining one of the main challenges in the biology of this tissue. Part of the problem is the enamel forming cells themselves, the ameloblasts, as they are non-proliferative and difficult to isolate and culture successfully. Ameloblasts also engage in a number of morphological changes across developmental stages concomitant with changes in their genetic profile adding to the complexity of the system. Yet they remain a particularly attractive model for potentially decoding relevant $Ca^{2+}$ signatures in mineralizing systems specifically, and as a unique cell $Ca^{2+}$ case study.

Enamel is the white outer cover of the tooth crown. In fully mineralized enamel, $Ca^{2+}$ is the main ionic species represented in the crystal structure. Thus much attention has been centred on the extracellular role of $Ca^{2+}$ as a mineralizing agent. $Ca^{2+}$ research in enamel biology indeed offers a wealth of possibilities to assess the versatility of this cation. This review will define the signalling role of $Ca^{2+}$ in enamel providing an overview of the different models proposed for the transport of $Ca^{2+}$ and how these might be redefined in light of recently reported data.

## Amelogenesis

Amelogenesis is a term used to describe both the formation (volume of tissue) and mineralization of enamel. Tooth enamel is a bioceramic of complex design and a prime example of cell mediated apatitic crystal formation via

matrix–crystal interactions. Mineralized enamel is four times harder than bone.

Ameloblasts derive from ectodermal epithelium and tightly control amelogenesis, a process commonly divided into two main stages termed secretory and maturation stages (Fig. 1). Secretory ameloblasts are polarized tall (~70 $\mu$m) post-mitotic cells with a specialized process at the distal end (see below) known as the Tomes'' process that plays a key role in enamel matrix secretion and the formation of enamel microstructure (Boyde, 1989). Secretory ameloblasts synthesize and secrete a protein-rich scaffold formed by a core of enamel matrix proteins (EMPs) that is subsequently removed during maturation leaving behind a highly organized biomaterial. Morphologically, secretory ameloblasts undergo a number of transformations losing the Tomes' process and reducing their height by about 50% to become maturation stage ameloblasts. The relevance of these changes can be more clearly appreciated when the histological transformations in cell anatomy and concomitant reorganization of organelle distribution occurring throughout amelogenesis are observed (see Fig. 1). This is even more significant when one considers the full spectrum of the functions associated with each cell stage.

During the maturation stage, the relatively long and thin crystals that had formed previously now increase to their full width and thickness, abutting each other almost entirely, a process facilitated by the increased activity of ameloblasts to transport ions and the removal of matrix (Robinson, 2014) (Fig. 1). Maturation stage ameloblasts are shorter (~40 $\mu$m) with an enlarged nucleus more centrally placed, and intercellular spaces increase. The most characteristic change at this stage is the switch from a cell with an apical ruffled (also called striated) border (RA) that is reformed into a smooth-ended border (SA) (Fig. 1) in a series of cycles impacting on ionic transport (Josephsen & Fejerskov, 1977; McKee *et al.* 1989). The reasons for this change are poorly understood.

## Enamel: matrix proteins and crystal growth

Fully formed enamel is an acellular tissue containing tightly packed crystallites and is the hardest and most durable vertebrate tissue containing ~95% mineral by weight (Smith, 1998).

**Enamel matrix proteins.** The composition of the proteinaceous scaffold provided by ameloblasts and its exact role in promoting crystal growth is still a subject of debate. The main protein secreted by ameloblasts (~90%) is amelogenin (AMEL) (Termine *et al.* 1980), an unstable 25 kDa hydrophobic protein cleaved by the matrix metalloprotease 20 (MMP20) (Robinson *et al.* 1998). It is commonly considered that AMEL self-assembles into nanospheres that adsorb to apatite inhibiting lateral crystal growth (Fincham *et al.* 1994; Brookes *et al.* 1995; Moradian-Oldak, 2001). Alternatively, in the presence of both $Ca^{2+}$ and phosphate in *in vitro* conditions under controlled pH, AMEL self-assembles into structures called nanoribbons which have the capacity to align into structures some micrometres long resembling enamel crystals (Martinez-Avila *et al.* 2011, 2012). Other proteins considered part of the unique ensemble of matrix proteins manufactured by ameloblasts include ameloblastin (AMBN) and enamelin (ENAM) which also show, albeit with considerable quantitative differences relative to AMEL, a pattern characterized by highest expression during the secretory stage (Smith & Nanci, 1996). Collectively, these proteins are regarded as structural proteins as they are involved at varying levels in determining the correct microstructure of enamel. In the maturation stage, the serine protease kallikrein 4 (KLK4) substitutes MMP20 for the proteolytic processing of peptides. Mutations to the genes encoding for *AMELX, AMBN, ENAM, MMP20 or KLK4* result in amelogenesis imperfecta, a term used to clinically describe a broad range of abnormal enamel phenotypes (Hart *et al.* 2000; Gibson *et al.* 2001; Paine *et al.* 2003; Wright *et al.* 2003; Kim *et al.* 2005).

It should be highlighted that enamel formation is clearly not a process that solely depends on $Ca^{2+}$ supply. Other important factors such as local extracellular pH, which is not stable throughout amelogenesis, has a major impact on crystal growth (Lacruz *et al.* 2010). Extracellular pH is heavily modulated by the activity of ameloblasts (Lacruz *et al.* 2010). Moreover, hydroxyapatite-like crystals formed *de novo* in the extracellular environment require the presence of calcium's largest partner, phosphate, to be able to generate the intial stages of crystal growth. However, this review is intended to focus on aspects of $Ca^{2+}$ transport and signalling in ameloblasts.

**Enamel crystal formation.** Thousands of individual crystals (~50 nm in diameter) are bundled into larger structures known as prisms of some 5 $\mu$m in diameter. Enamel prisms, each formed by a single ameloblast, are considered the basic microstructural unit of enamel (Boyde, 1989) (Fig. 2). Enamel crystals are seeded within the enamel fluid during the secretory stage in close contact with the apical end of the cell (Fig. 1). Analysis of pig enamel fluid showed that it differed in its composition from serum showing lower total $Ca^{2+}$ concentration ($[Ca^{2+}]$ was $10^{-3}$ M for serum and $10^{-4}$ M for enamel) supporting the notion that it represents a specialized micro-compartment (Aoba & Moreno, 1987). In the secretory stage, the bulk of $Ca^{2+}$ in the enamel fluid appears to be non-ionic with as much as 85% of the total $Ca^{2+}$ bound, possibly to AMEL-derived products (Moreno & Aoba, 1987; Robinson *et al.* 1998). Crystal formation requires supersaturation of the enamel fluid

by ions in secretory and maturation stages although the stoichiometry of the fluid and that of the minerals at each stage are different (Aoba, 1996). As discussed above, enamel is a highly mineralized biomaterial containing 'hydroxyapatite-like' crystals that is best considered as a non-stoichiometric carbonated $Ca^{2+}$ hydroxyapatite also incorporating ions, such as $Na^{+}$, $Mg^{2+}$, $Cl^{-}$ and $Fe^{3+}$, which compete for space in the crystal lattice and thus influence the properties of enamel (Young, 1974; Aoba, 1996). By weight, $Ca^{2+}$ represents about 36% of the minerals contained in matured enamel as measured in dried samples, about twice as much as the next ion species represented (Aoba & Moreno, 1987). Much of this $Ca^{2+}$ is incorporated during the maturation stage as the thin enamel crystals seeded during the preceding secretory stage now expand in width and thickness filling the spaces previously occupied by fluids and organics. Smith reports that about 86% of the $Ca^{2+}$ found in enamel enters the tissue during maturation stage (Smith, 1998).

## $Ca^{2+}$ transport in enamel

Changes in systemic $Ca^{2+}$ can directly impact on enamel. For example, enamel cells express the vitamin D receptor

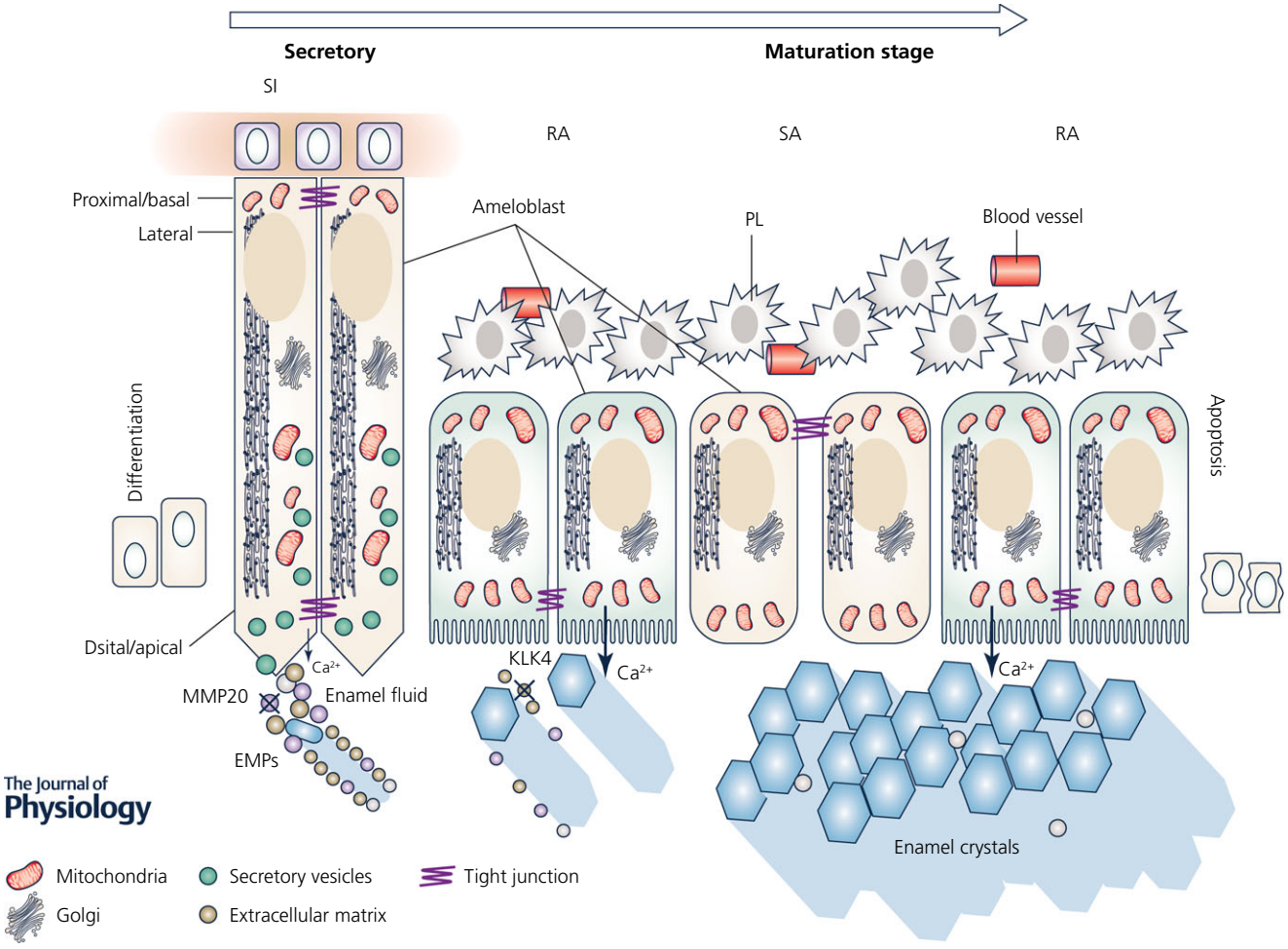


**Figure 1. Schematic diagram of histological changes in amelogenesis**
The histological development of enamel crystals goes hand in hand with changes in ameloblast morphology. Undifferentiated epithelial cells receive signals to transform into secretory ameloblast cells of some 75 $\mu$m tall and ~5 $\mu$m in diameter with a specialized distal cell process (Tomes' process) which plays an important role in matrix exocytosis. These same cells will retransform into shorter cells (~35 $\mu$m tall) during maturation devoid of the Tomes' process. In maturation stage, ameloblasts undergo cyclical changes from a cell with a distal ruffled border, the ruffled-ameloblast (RA), to a cell with a smooth distal border, the smooth-ameloblast (SA). Tight junctions are found at the basal and apical pole of secretory ameloblasts. The apical or distal pole is closest to the enamel crystals. In RA cells, tight junctions are found only at the apical pole but in SA cells they are located at the basal pole. Organellar distribution differs in cells at each stage (see text for details). SI = stratum intermedium, PL = papillary layer, EMPs = enamel matrix proteins. MMP20 and KLK4 are the main proteases in AMEL processing. See also organellar distribution at each stage.

(VDR; Davideau *et al.* 1996) and in conditions of normal calcaemia, low vitamin D levels result in several abnormal dental phenotypes highlighting the role of systemic $Ca^{2+}$ levels in the formation of dental enamel (Berdal *et al.* 1993). It also underscores its relevance for understanding $Ca^{2+}$ transport by enamel epithelium.

The mechanisms involved in $Ca^{2+}$ transport by enamel organ cells are far from clear (Bawden, 1989; Takano, 1995; Smith, 1998; Hubbard, 2000) and many of the methods used to report differences in $Ca^{2+}$ dynamics are outdated (see below). The proposed models, however, agree that $Ca^{2+}$ incorporation into enamel arises not from the underlying mesenchymal zone of the dentine, but from the enamel organ itself (Reith & Boyde, 1978). The main implication is that $Ca^{2+}$ travels in the basolateral to apical direction and across the barrier formed by ameloblast cells (Fig. 1). Whether this transport occurs via an active transcellular route or passively across intercellular spaces (paracellular transit) has been the subject of a number of studies.

Because secretory and maturation stage ameloblasts are organized in cell cohorts with each cell bound to its neighbouring cell by tight junctions (Fig. 1), there are important constraints on the paracellular/intercellular or passive movement of ions to the forming enamel. Lanthanum tracer studies showed that it penetrated the proximal but not the distal intercellular junctions of secretory ameloblasts (Takano & Crenshaw, 1980), whereas injections of radiolabelled $Ca^{2+}$ ($^{45}Ca$) in 6-day old rats suggested an intercellular route (Hanawa *et al.* 1990). Secretory stage ameloblasts stained with glyoxal bis(2-hydroxyanil) (GBHA) did not find evidence of staining in the intercellular spaces but found strong reactions within the cells (Takano *et al.* 1989).

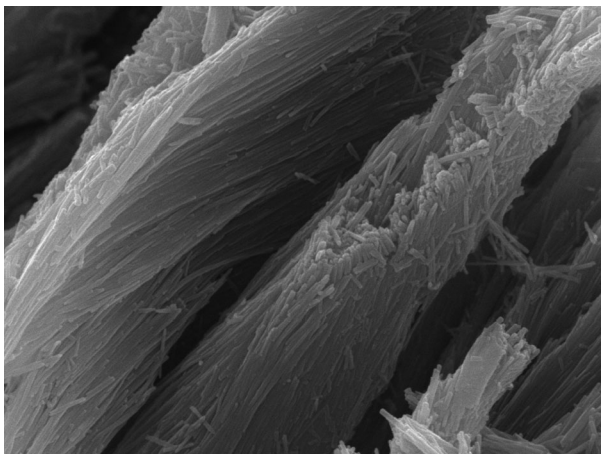

**Figure 2. Electron micrograph showing enamel crystals and prisms**
Enamel crystals are needle-like structures that elongate for hundreds of micrometres. These crystals are formed by accumulation of $Ca^{2+}$ and phosphate. Thousands of these crystals are bundled forming a prism, which is the basic microstructural unit of mineralized enamel. The proper development of crystals and prisms are modulated by ameloblasts and their products. Field-width is approximately 12 microns.

In maturation ameloblasts, tight junctions reorganize their localization. In the predominant ruffled-ended phase, tight junctions are only found near the basolateral pole, this being reversed in the smooth-ended phase with junctions found apically (Fig. 1) (Josephsen & Fejerskov, 1977). Regardless, neither lanthanum nor horseradish peroxidase (HRP) could penetrate across the distal junctions of ruffled ameloblasts or the proximal junctions of smooth-ended cells (Takano & Crenshaw, 1980; Takano, 1995). This cellular reorganization consisting of ruffled-to-smooth waves is a system available only to ameloblast cells and seems to be advantageous to these cells. In the smooth-ended phase, ameloblasts appear to rely on intracellular transport. Physiological limitations imposed in this system include the relatively long period of time between the two maturation stage cell morphologies, and that about 70% of all maturation ameloblasts are ruffled-ended (Josephsen & Fejerskov, 1977; Smith, 1998). $^{45}Ca$ studies suggest that $Ca^{2+}$ is incorporated in bulk through the ruffled-border stage (Takano & Crenshaw, 1980; Reith & Boyde, 1981; McKee *et al.* 1989). Although aptly pointed out by Smith that ameloblasts appear to optimally utilize both the gated (ruffled) and non-gated (smooth) ionic transport system (Smith, 1998), reviewing the data discussed above and largely based on the lack of diffusion of lanthanum and HRP across intercellular junctions, Bawden, Takano and Hubbard strongly suggested that the dominant paradigm for the transport of $Ca^{2+}$ was via a transcellular route (Bawden, 1989; Takano, 1995; Hubbard, 2000). Given the wider acceptance now of a dominant active $Ca^{2+}$ transport system by the enamel organ, this in turn requires a number of molecular mechanisms common to most cells involving intracellular $Ca^{2+}$ stores, release channels that enable moderate increases in $[Ca^{2+}]_i$ and a $Ca^{2+}$ influx system. Here we examine the available evidence.

## $Ca^{2+}$ release via stores

Cytoplasmic $Ca^{2+}$ increase can occur either from the release of stored $Ca^{2+}$ in the luminal compartments of a number of intracellular organelles, or from influx of $Ca^{2+}$ from outside of the cell. While the majority of intracellular $Ca^{2+}$ is stored in endoplasmic reticulum (ER; ~1 mM; Prins & Michalak, 2011) or sarcoplasmic reticulum (SR) in muscle cells, intracellular $Ca^{2+}$ is also found in mitochondria, endolysosomal compartments, Golgi apparatus and peroxisomes (Prins & Michalak, 2011).

Release of $Ca^{2+}$ from the ER/SR can be controlled by $Ca^{2+}$ itself, or by a group of intracellular messengers such as inositol-1,4,5-trisphosphate ($IP_3$), cyclic ADP ribose

(cADPR), nicotinic acid adenine dinucleotide phosphate (NAADP) and sphingosine-1-phosphate (S1P), which either stimulate or modulate $Ca^{2+}$ release. The most common channels associated with the ER are inositol 1,4,5-trisphosphate receptors ($IP_3Rs$) and/or ryanodine receptors (RyRs) (Fill & Copello, 2002; Stathopulos *et al.* 2012).

**Inositol receptors.** Binding of the G-coupled receptor on the cell membrane to its ligand induces tyrosine phosphorylation and activation of phospholipase C (PLC). PLC hydrolyses the phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)$P_2$ or $PIP_2$), a component of cell membranes, to release soluble $IP_3$ and diacylglycerol (DAG). Within seconds, $IP_3$ binds to its receptor known as $IP_3R$ located on the surface of the ER increasing $[Ca^{2+}]_i$ (Wagner & Yule, 2012). The $IP_3R$ channel consists of large proteins (300 kDa) (Hajnoczky *et al.* 2000; Szabadkai *et al.* 2006). Three predominant $IP_3R$ isoforms have been described ($IP_3R1$, $IP_3R2$ and $IP_3R3$) in vertebrates with 60–80% homology in their amino acid sequences (Shah *et al.* 2015). $IP_3Rs$ are encoded by several genes with a number of isoforms formed through splicing (Foskett *et al.* 2007; Mikoshiba, 2007). A number of disease states have been described associated with the different $IP_3R$ subtypes (Mikoshiba, 2015). $IP_3R1$ deficiency causes abnormal fertilization and severe neurological disorders (Miyazaki *et al.* 1992; Matsumoto *et al.* 1996; Foskett, 2010; Higo *et al.* 2010). $IP_3R2$ overexpression results in hypertrophy of cardiac muscle (Nakayama *et al.* 2010) whereas $IP_3R2$ mutations inhibit sweat secretion (Klar *et al.* 2014). $IP_3R3$ is important in taste perception, hair growth and osteoclast formation.

**Ryanodine receptors.** RyRs are high-conductance, cation selective, tetrameric ligand-gated $Ca^{2+}$ release channels that mediate $Ca^{2+}$ release from the SR essential for muscle contraction. The three known mammalian isoforms (RyR1, RyR2 and RyR3) display a high degree of similarity in the peptide sequence and three-dimensional structure (Baker *et al.* 2015). RyR1 is predominantly expressed in skeletal muscle and in cerebellar Purkinje neurons (Takeshima *et al.* 1989; Zorzato *et al.* 1990; Furuichi *et al.* 1994; Hertle & Yeckel, 2007). RyR2 is the most abundant isoform in the brain and is greatly expressed in cardiac muscle (Nakai *et al.* 1990; Otsu *et al.* 1990; Lai *et al.* 1992; Hertle & Yeckel, 2007). RyR3 was first identified in the brain and is mainly found in cortical and hippocampal regions involved in learning and memory (Hakamata *et al.* 1992; Futatsugi *et al.* 1999; Hertle & Yeckel, 2007). RyR3 is also expressed in the diaphragm (Marks *et al.* 1989). Several mutations in both RyR1 and RyR2 are associated with human disorders such as malignant hyperthermia (Denborough, 1998) and later central core disease, catecholaminergic polymorphic ventricular tachycardia, and arrhythmogenic right ventricular dysplasia (Lanner *et al.* 2010).

**$IP_3Rs$ and RyRs in enamel cells.** $IP_3Rs$ were first identified in dental enamel cells using radioligand binding (Hubbard, 1996). However, the cellular localization, isoforms and potential difference in expression level in secretory and maturation stage cells were not reported (Hubbard, 1996). Our group reported recently that the mouse-derived ameloblast-like cell line, LS8 cells (Chen *et al.* 1992), express $IP_3Rs$ and RyRs at the mRNA and protein level, $IP_3R3$ and RyR2 being the predominant homologues in this cell line (Nurbaeva *et al.* 2015*b*). In primary cells, $IP_3R$ expression was detected in secretory and maturation stage rat ameloblasts at considerably higher levels than RyR expression. Immunofluorescence analysis by confocal microscopy revealed differences in cellular localization for $IP_3Rs$ with $IP_3R1$ and $IP_3R3$ found largely in the cytoplasm and $IP_3R2$ was found only in the cell nuclei of both secretory and maturation enamel organ cells (Nurbaeva *et al.* 2015*a*). As RyRs are most commonly identified in excitable cells (Bennett *et al.* 1996), it is likely that ER $Ca^{2+}$ release is mediated by $IP_3Rs$ in ameloblasts and LS8 cells but this has yet to be assessed in more detail.

## Store operated $Ca^{2+}$ entry

Depletion of ER $Ca^{2+}$ stores activates store-operated $Ca^{2+}$ entry (SOCE) channels (Prakriya & Lewis, 2015). The best characterized SOCE channels that are functional in many cell types and organs are $Ca^{2+}$ release-activated $Ca^{2+}$ (CRAC) channels, which are highly $Ca^{2+}$ selective (Prakriya & Lewis, 2015). CRAC channels are composed of ORAI subunits, which belong to a small family of conserved integral plasma membrane (PM) proteins that form the pore of the CRAC channel. The best characterized family member is ORAI1, but ORAI2 and ORAI3 may also contribute to SOCE in certain tissues. CRAC channels are activated by two proteins located in the membrane of the ER, stromal interaction molecule (STIM) 1 and STIM2, which sense the ER $Ca^{2+}$ concentration and bind to ORAI proteins upon $Ca^{2+}$ depletion from the ER to enable sustained $Ca^{2+}$ entry (Shaw *et al.* 2013).

**ORAI and STIM proteins.** STIM1 and its homologue STIM2 are single-pass transmembrane proteins located in the ER with a luminal N-terminus and a cytosolic C-terminus (Shaw *et al.* 2013; Prakriya & Lewis, 2015). In non-stimulated cells, the $Ca^{2+}$ concentration in the ER is high (in the same range as that in the extracellular medium, ~1 mM) and $Ca^{2+}$ is bound to a canonical EF-hand $Ca^{2+}$ binding domain in the N-terminus of STIM1 and STIM2 which is located proximal to a sterile alpha motif (SAM). The STIM C-terminus contains three

coil-coil (CC) domains and a polybasic region (Prakriya & Lewis, 2015). The second and third CC domains form a domain variously known as CRAC channel activation domain (CAD) (Park *et al.* 2009), STIM-ORAI activating region (SOAR) (Yuan *et al.* 2009) or CC fragment b9 (CCb9) (Kawasaki *et al.* 2009) that is necessary and sufficient for CRAC channel activation (Fig. 3*A*). ORAI1 is a tetraspanning PM protein that assembles in a hexameric protein complex and forms the pore of the CRAC channel (Fig. 3*A*) (Feske *et al.* 2006; Prakriya *et al.* 2006; Vig *et al.* 2006; Yeromin *et al.* 2006). ORAI1 contains intracellular N- and C-termini through which it interacts with STIM1 and STIM2 as well as several other proteins that were shown to regulate CRAC channel function and SOCE (Prakriya & Lewis, 2015).

**$Ca^{2+}$ entry in enamel cells via CRAC channels.** In the past, $Ca^{2+}$ influx into ameloblasts has been largely considered a passive event (Bawden, 1989; Takano, 1995; Hubbard, 2000). Our recent work elucidated new aspects of the $Ca^{2+}$ entry process implicating CRAC channels as key modulators. (Nurbaeva *et al.* 2015*a*,*b*). Our data emanated from a genome wide study in which we compared rat enamel organ cells from the maturation and secretory stage (Lacruz *et al.* 2012*a*). These genomic data identified *Stim1* and *Stim2* transcripts as being up-regulated in maturation, which was confirmed by Western blot analysis (Lacruz *et al.* 2012*a*). While STIM1 and STIM2 proteins were expressed in the enamel organ, little was known about their putative function in this system. A few years earlier, we had reported that patients with loss-of-function or null mutations in *STIM1* and *ORAI1* genes present with a hypocalcified form of amelogenesis imperfecta (McCarl *et al.* 2009; Picard *et al.* 2009; Fuchs *et al.* 2012), which strongly suggested that both proteins and thus CRAC channels are important in enamel formation. However, physiological data demonstrating CRAC channel activity in enamel cells was missing. To directly demonstrate a role of CRAC channels in dental enamel, we first used ameloblast-like LS8 cells to develop and test protocols used in many other cells to investigate SOCE via the CRAC channel. Passive depletion of ER $Ca^{2+}$ stores of LS8 cells with thapsigargin, an inhibitor of the sarco/endoplasmic reticulum ATPase (SERCA), followed by re-addition of

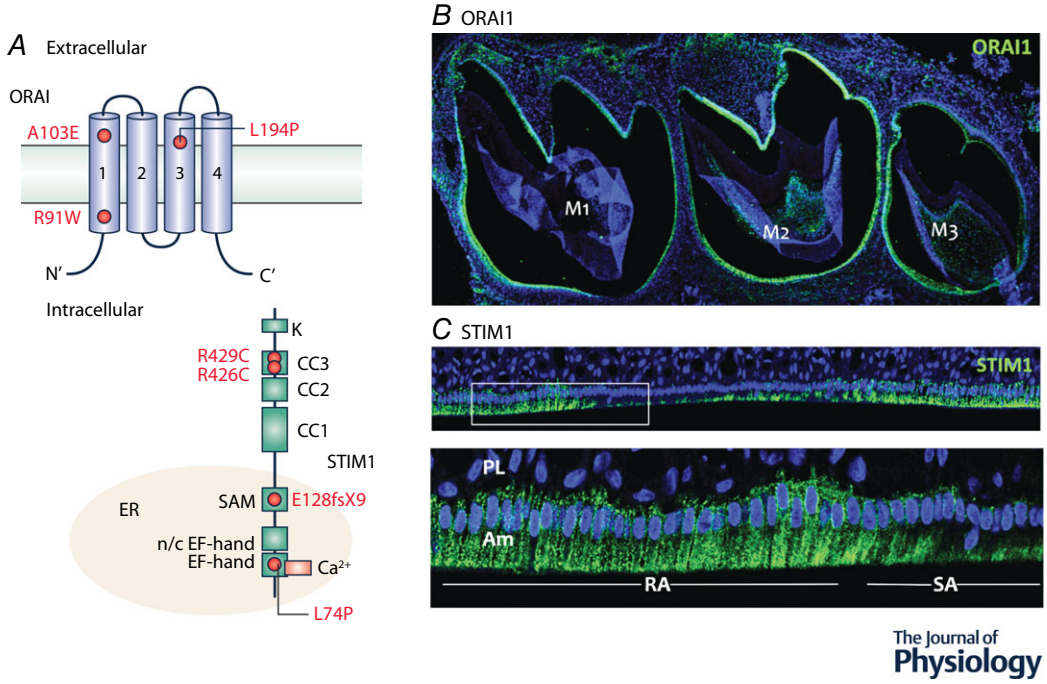


**Figure 3. STIM1 and ORAI1 mutations affecting enamel and localization in ameloblasts**
*A*, diagrammatic representation of STIM1 and ORAI1 protein structure. STIM1 is a single pass membrane in the endoplasmic reticulum (ER) with the N-terminus found in the ER lumen. ORAI1 is a plasma membrane (PM) bound protein with four transmembrane domains. Both N- and C-termini are cytosolic. The red dots in each protein mark the known mutations at each domain impacting enamel development. ER = endoplasmic reticulum, SAM = sterile alpha motif, CC = coil-coil domain. *B*, ORAI1 localization by immunofluorescence microscopy in mouse molar (M) ameloblasts showing limited distribution of this protein. *C*, STIM1 localization in rat ameloblasts. Immunofluorescence staining shows differences in STIM1 localization between ruffled-ameloblasts (RA) and smooth-ameloblasts (SA). In RA cells STIM1 localizes throughout the cytosol whereas in SA cells, STIM1 signals markedly decrease suggesting a more important role for STIM1 during the RA stage. In *B* and *C* DAPI is shown in blue. Am = ameloblasts, PL = papillary layer.

extracellular $Ca^{2+}$ to the cells resulted in a marked increase in $[Ca^{2+}]_i$ due to SOCE. By contrast, LS8 cells treated with a number of inhibitors (Synta 66, 2-APB, BTP2) that have been used to suppress CRAC channel function lacked the thapsigargin-induced $[Ca^{2+}]_i$ increase, indicating that ameloblast-derived cells indeed have functional CRAC channels and SOCE. This conclusion is supported by data showing that the major protein components of the CRAC channel, i.e. STIM1, STIM2, ORAI1, ORAI2 and ORAI3, as well as SERCA2, which mediates reuptake of $Ca^{2+}$ into the ER, are expressed in these cells (Nurbaeva *et al.* 2015*b*).

To confirm that CRAC channel components are present in primary enamel cells and to investigate their expression throughout the development of the enamel organ, we studied dental tissues from mice and rats by RT-PCR and immunofluorescence staining. ORAI1, ORAI2 and ORAI3 as well as STIM1 and STIM2 mRNAs are all expressed in rodent teeth at both the maturation and secretory stages of enamel development (Nurbaeva *et al.* 2015*a*). We noted that mRNA expression of all ORAI and STIM homologues increased in maturation stage consistent with an increased $Ca^{2+}$ transport function at this stage. mRNA analysis showed that ORAI1 was the predominant ORAI isoform (see also Fig. 3*B* for protein expression) and as expected, its expression in ameloblasts was enriched at the PM of RA cells consistent with its role as a pore-subunit of the channel (Nurbaeva *et al.* 2015*a*). Uncharacteristically, another study reported a cytosolic expression of ORAI1 in ameloblasts (Zheng *et al.* 2015). We found that STIM1 was localized throughout the cytosol of maturation stage ameloblasts (Nurbaeva *et al.* 2015*a*) as also reported by Simmer and Hu's group (Wang *et al.* 2014). Further analysis showed that this was the case only in RA cells whereas SA cells showed significantly decreased immunoreactivity (Fig. 3*C*) suggesting that $Ca^{2+}$ uptake via CRAC channels may predominantly occur in RA cells. Although STIM2 mRNA expression was somewhat more abundant than that of STIM1 in ameloblasts isolated at the maturation stage, at the protein level we had previously reported that STIM1 protein levels are higher than those of STIM2 (Lacruz *et al.* 2012*a*).

The isolation of enamel organ cells from the secretory or maturation stages was originally reported by Smith and Nanci (Smith & Nanci, 1989). Using their method to manually separate each cell type enabled us to directly investigate SOCE in primary enamel organ cells after stimulation with thapsigargin to passively deplete ER $Ca^{2+}$ stores and to induce SOCE. Cells from both stages of enamel development showed $Ca^{2+}$ release from the ER when treated with thapsigargin, and a strong increase in $[Ca^{2+}]_i$ following re-addition of $Ca^{2+}$ to the extracellular bath solution, indicating that both cell types have functional SOCE. Supporting this conclusion, $Ca^{2+}$ entry was severely inhibited when secretory and maturation stage ameloblasts were pre-treated with the CRAC channel inhibitor Synta 66 (Nurbaeva *et al.* 2015*a*) and 2-aminoethyldiphenyl borinate (2-APB) (author's unpublished observations) demonstrating that CRAC channels mediate SOCE in enamel cells. It should be pointed out that neither Synta 66 nor 2-APB completely abrogated $Ca^{2+}$ entry as there was a small increase in $[Ca^{2+}]_i$. It might be possible that other $Ca^{2+}$ channels also contribute to $Ca^{2+}$ influx in enamel cells. Some members of the transient receptor potential (TRP) channel family have been detected in pre-secretory ameloblasts (Liu *et al.* 2015). However, direct functional evidence supporting a role of these channels in $Ca^{2+}$ influx is missing and the potent inhibition of $Ca^{2+}$ influx in ameloblasts using CRAC channel blockers suggests that SOCE is a dominant pathway for $Ca^{2+}$ influx in enamel cells.

**Mutations in *STIM1* and *ORAI1* cause amelogenesis imperfecta in patients.** Homozygosity for autosomal recessive loss-of-function or null mutations in *STIM1* or *ORAI1* genes that abolish CRAC channel function and SOCE is associated with hypocalcified *amelogenesis imperfecta* (McCarl *et al.* 2009; Picard *et al.* 2009; Fuchs *et al.* 2012; Lacruz & Feske, 2015). For ORAI1, a number of mutations in different regions of the protein (R91W, Feske *et al.* 2006; A103E, L194P and A88SfsX25, McCarl *et al.* 2009; see also Fig. 3*A*) have been recognized that cause severe, often lethal immune dysfunction. The dental phenotype of ORAI1-deficient patients is similar in most patients being characterized by dysplastic enamel. The photographs of the patients' teeth show a high degree of enamel attrition in both primary and secondary teeth with dentine exposure and enamel discolouration (McCarl *et al.* 2009).

The first reported null mutation in *STIM1* associated with an enamel defect was identified in three siblings homozygous for a frameshift mutation in the N-terminus of *STIM1* (E128RfsX9 (or E136X)) (Fig. 3*A*) (Picard *et al.* 2009). Another *STIM1* mutation associated with an enamel defect was identified in a patient homozygous for a missense mutation located in the SOAR/CAD/CCb9 domain of STIM1 (R429C) (Fuchs *et al.* 2012). The black and white oral photographs of this patient (reported in Fuchs *et al.* 2012) suggest that his teeth may have preserved residual enamel but they show advanced wear in all teeth. More recently, another patient with a homozygous missense mutation in the SOAR/CAD/CCb9 domain of STIM1 (R426C) was reported (Wang *et al.* 2014). The dental phenotype of this patient has been described to include enamel attrition with cream-coloured or brown teeth, although the patient's teeth were of similar size and shape as those of a control (Wang *et al.* 2014). Intriguingly, whereas the patient with R429C mutation suffered from the full clinical phenotype associated with CRAC channelopathy, including immunodeficiency,

muscular and enamel dysplasia (Fuchs *et al.* 2012), the patient with R426C mutation only presented with an enamel defect (Wang *et al.* 2014), suggesting that his mutation may be a hypomorph and that some STIM1 function and SOCE are preserved. The most recently reported missense mutation in *STIM1* results in the substitution of leucine 74 in the STIM1 EF-hand domain with proline (L74P) (Parry *et al.* 2015) and was found in two teenaged cousins. The *STIM1* p.L74P mutation resulted in hypomineralized amelogenesis imperfecta with discoloured enamel in both primary and secondary teeth (Parry *et al.* 2015).

Collectively, these data demonstrate that loss-of-function or null mutations in *STIM1* or *ORAI1* that impair SOCE result in rapid wear of enamel with discolouration of teeth and exposure of the underlying dentine, whereas the shape and size of teeth appeared to be normal. The advanced enamel wear reported in all patients strongly implies that a major effect of the mutations is hypomineralized enamel. However, all of these studies are based solely on oral photographs and in some cases dental X-rays. Because of the limited availability of patient tissue, they do not allow an in-depth analysis of the enamel tissue or possible changes in ameloblast function or morphology, and they have not investigated whether $Ca^{2+}$ entry is altered in patient ameloblasts.

It is noteworthy that no mutations in *STIM2*, *ORAI2* or *ORAI3* genes have been reported to date in human patients, but this does not exclude a potential role of these genes in SOCE and enamel development.

## Signalling role of $Ca^{2+}$ in enamel

The bulk of data available on $Ca^{2+}$ transport by enamel epithelium reflect the overwhelming interest in the role of secreted $Ca^{2+}$ and its incorporation into mineral deposits. Yet many of the processes that temporally precede mineralization remain poorly understood. Among these, is the intriguing concept that $Ca^{2+}$ may act as a secondary messenger which modulates a number of processes during amelogenesis including the regulation of enamel protein expression.

It is well known that a rise in $[Ca^{2+}]_i$ has broad versatility in cell signalling (Berridge *et al.* 2003) and thus we investigated the potential effects of a CRAC channel mediated $[Ca^{2+}]_i$ rise in the expression of enamel genes. We found that stimulating LS8 cells with thapsigargin for 30 min in the presence of extracellular $Ca^{2+}$, the mRNA levels of the main enamel genes (*Amelx, Ambn, Enam*) significantly increased. This effect was reversed if LS8 cells were pre-treated with 2-APB, a CRAC channel inhibitor (Nurbaeva *et al.* 2015*b*), indicating the SOCE-mediated $Ca^{2+}$ entry impacted enamel gene expression in LS8 cells. The same positive effect in the expression of enamel genes was found in primary enamel cells dissected from mouse enamel organs stimulated with thapsigargin and although here we did not test these cells using inhibitors, the fact that we observed a positive response after passive depletion of ER $Ca^{2+}$ stores using thapsigargin is indicative of SOCE involvement in mediating the expression of enamel genes (Nurbaeva *et al.* 2015*b*). Changes in gene expression were also assessed at other time points (1 h, 1.5 h) in LS8 cells but these showed only a moderate up-regulation of mRNA for these genes. These data suggest that modulation of enamel genes by SOCE is very sensitive. Protein changes analysed by Western blot showed that AMBN expression increased in primary cells after 1 h of stimulation with thapsigargin, supporting the relatively fast action of SOCE on enamel protein expression. Using a small interfering (si)RNA knock-down approach in HAT-7 ameloblast-like cells, others have also shown that in cells transfected with an siRNA against *ORAI1*, *Amelx* and *Ambn* expression declined supporting the role of CRAC channels in modulating enamel gene expression (Zheng *et al.* 2015).

An increase in $[Ca^{2+}]_i$ can also occur in response to $Ca^{2+}$ discharge from intracellular stores into the cytosol. Our preliminary unpublished data suggest that thapsigargin stimulation by itself in the absence of $Ca^{2+}$ does not impact the mRNA levels of enamel genes indicating that the changes in gene expression described above are the result of SOCE activation and is not due to release from intracellular stores.

## $Ca^{2+}$ sensing molecules

Previous *in vitro* studies have shown that exposure of the immortalized ameloblast-like cell line PABSo-E cells to extracellular $Ca^{2+}$ at various concentrations had a positive effect on the expression of *AMELX* and other enamel genes, and mediated ameloblast cell differentiation (Bronckers *et al.* 2006; Chen *et al.* 2009). The $Ca^{2+}$ sensing receptor (CaSR) was identified in the PABSo-E cell line suggesting that CaSR might be a mechanism used by these cells to interpret changes in extracellular $Ca^{2+}$ to regulate cell function (Mathias *et al.* 2001). Exposure of PABSo-E cells to varying concentrations of extracellular $Ca^{2+}$ resulted in a concentration-dependent rise of $[Ca^{2+}]_i$ measured by Fura-2 (Mathias *et al.* 2001). CaSR is indeed involved in the regulation of diverse cellular processes including ion channel activity via activation of PLC, which is a common pathway involved in ER $Ca^{2+}$ release via $IP_3$Rs and the activation of SOCE (Ye *et al.* 1996, 1997; Chattopadhyay *et al.* 1998). Thus, the extracellular $Ca^{2+}$ concentration, sensed by CaSR, could plausibly regulate the uptake of $Ca^{2+}$ into enamel cells via activation of SOCE. However, unpublished data from our lab detected only a minimal rise in $[Ca^{2+}]_i$ when primary enamel cells loaded with Fura-2 were subjected to a change in solution from 0 mM $Ca^{2+}$ to 2 mM $Ca^{2+}$.

## $Ca^{2+}$ binding proteins (CaBPs)

**Cytoplasmic $Ca^{2+}$ binding proteins.** Hundreds of cellular proteins have been adapted to bind $Ca^{2+}$, acting either as $Ca^{2+}$ sensors or buffers. $Ca^{2+}$ buffers generally do not undergo major conformational changes upon binding to $Ca^{2+}$ and comprise only a small subset of cytosolic proteins of the EF-hand family including parvalbumins, calbindin 9kDa, calbindin 28kDa and calretinin. The majority of EF-hand proteins belong to the group of $Ca^{2+}$ sensors. Binding of $Ca^{2+}$ to $Ca^{2+}$ binding sensor protein induces conformational changes enabling them to interact with specific targets. Prototypical examples of $Ca^{2+}$ sensors are calmodulin (Chin & Means, 2000), calcineurin (a $Ca^{2+}$/calmodulin-dependent protein phosphatase) and some of the proteins from the S100 protein family. However, $Ca^{2+}$ sensors may also function as $Ca^{2+}$ buffers.

Parvalbumins. Parvalbumin is an EF-hand type $Ca^{2+}$-binding albumin protein with molecular weight of 12 kDa (gene symbol: *PVALB*). This protein is divided into three domains each containing an EF-hand (Swain *et al.* 1989). The global role of palvalbumin is to serve as a cytosolic $Ca^{2+}$ buffer, while $Ca^{2+}$-dependent conformational changes can activate its $Ca^{2+}$ sensor function (Cox *et al.* 1999).

Localization of palvalbumin during different stages of enamel formation has been investigated using immunogold cytochemistry (Davideau *et al.* 1993). In early secretory ameloblasts, palvalbumin distribution was limited, being found in association with the Tomes' process. In older secretory cells this pattern changed, becoming distributed throughout the cell. During the maturation stage, parvalbumin was more abundant in the central area of the cell and in the distal pole of RA cells than in corresponding areas of smooth-ended ameloblasts (Davideau *et al.* 1993).

Calbindin 9kDa and calbindin 28kDa. Calbindin 9kDa (gene symbol: *S100G*) is a protein with four $\alpha$-helical regions forming an EF-hand pair consisting of a canonical (EF2) and a non-canonical (EF1) domain (Kordel *et al.* 1993). Calbindin 9kDa undergoes $Ca^{2+}$-induced conformational changes but functions predominantly as a $Ca^{2+}$ buffer rather than as a $Ca^{2+}$ sensor (Skelton *et al.* 1994). Calbindin 28kDa (gene symbol: *CALB1*) has six EF-hand domains (Cheung *et al.* 1993). Calbindin 28kDa's $Ca^{2+}$-dependent conformational changes indicate additional $Ca^{2+}$ sensor functions (Berggard *et al.* 2002).

A number of studies reported the expression of calbindin 9kDa and calbindin 28kDa in ameloblasts of rat molars and incisors (Berdal *et al.* 1993, 1996; Hubbard, 1995, 1996; Turnbull *et al.* 2004; Kutuzova *et al.* 2006; Lee *et al.* 2007; Hubbard *et al.* 2011). For calbindin 9kDa, immunolocalization, radioimmunoassay and mRNA analysis showed higher levels during the maturation stage (Table 1) (Taylor *et al.* 1984; Berdal *et al.* 1991, 1993). In contrast, calbindin 28kDa expression level was higher in rat secretory ameloblasts (Table 1) (Hubbard, 1995). It has been suggested that fixation methods may influence the distribution of calbindin 9kDa and calbindin 28kDa seen by immunolabelling. Using this method, both calbindins localized in the nucleus and cytoplasm, but only calbindin 9kDa was observed in mitochondria in ameloblasts, as also described in other cells (Berdal *et al.* 1991). High expression levels of calbindin 9kDa during the maturation stage might suggest that calbindin 9kDa plays an important role during enamel maturation. However, calbindin 9kDa mutations do not result in any dental phenotype (Kutuzova *et al.* 2006; Lee *et al.* 2007). Moreover, calbindin 28kDa null mutant mice also lack a dental phenotype (Hubbard, 1995, 1996; Turnbull *et al.* 2004). Data from several sources identified that calbindin 9kDa and calbindin 28kDa gene expression appeared to be regulated by vitamin D in ameloblasts (Taylor, 1984; Taylor *et al.* 1984; Berdal *et al.* 1993), with these cells also expressing vitamin D receptors (Berdal *et al.* 1993).

Calretinin. Human calretinin (31 kDa; gene symbol: *CALB2*) consists of 271 amino acids and has six EF-hand domains, five of which are able to bind $Ca^{2+}$ (Schwaller *et al.* 1997; Stevens & Rogers, 1997). Calretinin $Ca^{2+}$-dependent conformational changes suggest that calretinin may also have $Ca^{2+}$-sensor functions (Billing-Marczak & Kuznicki, 1999).

In enamel cells, calretinin is expressed during the differentiation stage when generalized epithelial cells become pre-ameloblasts but no data have been reported for secretory or maturation stages (Hubbard *et al.* 2011). Mutations to calretinin do not appear to have a major impact in enamel as no dental phenotypes have been reported.

Calmodulin. Calmodulin (16 kDa; gene symbol: *CALM*) is a ubiquitous, highly conserved EF-hand containing a $Ca^{2+}$ binding domain. This protein localizes to the cytosol and to the nucleus (Maier & Bers, 2002) and binds up to four $Ca^{2+}$ via each EF-hand motif. Calmodulin undergoes $Ca^{2+}$-dependent conformational changes that increase its affinity for target proteins (Maier & Bers, 2002). Calmodulin is involved in many processes including growth, proliferation and also in the immune system. As a $Ca^{2+}$ sensor, calmodulin has the ability to detect and respond to a range of changes in $[Ca^{2+}]_i$ (Chin & Means, 2000). Using immunoblot analysis it has been shown

**Table 1. $Ca^{2+}$ signalling molecules in ameloblasts. Arrows indicate whether expression was high or low at each stage**

| Name of molecules | Ameloblasts: Secretory | Ameloblasts: Maturation | Methods | Mutation shows dental phenotype | Reference |
|---|---|---|---|---|---|
| Cytoplasmic $Ca^{2+}$ binding molecules | | | | | |
| (i) $Ca^{2+}$ buffers | | | | | |
| Parvalbumin | Same | Same | Protein expression | ? | (Davideau *et al.* 1993) |
| Calbindin-D9k | ↓ | ↑ | mRNA, protein expression | No | (Hubbard *et al.* 2011), (Berdal *et al.* 1993), (Berdal *et al.* 1996), (Kutuzova *et al.* 2006) |
| Calbindin-D28k | ↑ | ↓ | Protein expression | No | (Hubbard, 1995), (Berdal *et al.* 1993), (Berdal *et al.* 1996), (Turnbull *et al.* 2004) |
| Calretinin | Expressed during differentiation stage! | | Protein expression | No | (Hubbard *et al.* 2011), (Schurmans *et al.* 1997) |
| (ii) $Ca^{2+}$ sensors | | | | | |
| Calmodulin | Same | Same | Protein expression | ? | (Hubbard, 1995) |
| Calcineurin | Same | Same | — | ? | (Hubbard, 1995) |
| ER $Ca^{2+}$ binding proteins | | | | | |
| Calreticulin | ↓ | ↑ | Protein expression | ? | (Hubbard, 1996) |
| Endoplasmin | ↓ | ↑ | Protein expression | ? | (Hubbard, 1996) |
| ERp72 | ↑ | ↓ | Protein expression | ? | (Hubbard *et al.* 2000) |
| Calnexin | Same | Same | Immunofluorescence | ? | (Nurbaeva *et al.* 2015*a*) |
| ER $Ca^{2+}$ signalosomes | | | | | |
| STIM1 | ↓ | ↑ | mRNA, protein expression | Yes | (Lacruz *et al.* 2012*a*), (Wang *et al.* 2014; Nurbaeva *et al.* 2015*a*), (Picard *et al.* 2009; Feske, 2011) |
| STIM2 | ↓ | ↑ | mRNA, protein expression | No | (Lacruz *et al.* 2012*a*), (Nurbaeva *et al.* 2015*a*) |
| $IP_3R$ | — | — | Radioligand binding | — | (Hubbard, 1996) |
| $IP_3R1$, $IP_3R2$, $IP_3R3$ | Same | Same | Protein expression | ? | (Nurbaeva *et al.* 2015*a*) |
| RyR1,RyR2, RyR3 | Not expressed (?) | Not expressed (?) | mRNA, protein expression | ? | (Nurbaeva *et al.* 2015*a*) |

(*Continued*)

**Table 1. Continued**

| Name of molecules | Ameloblasts | | Methods | Mutation shows dental phenotype | Reference |
|---|---|---|---|---|---|
| | Secretory | Maturation | | | |
| $Ca^{2+}$ entry | | | | | |
| ORAI1 | ↓ | ↑ | mRNA, protein, function | Yes | (Feske, 2011; Nurbaeva *et al.* 2015*a*), (McCarl *et al.* 2009) |
| ORAI2 | ↓ | ↑ | mRNA, function(?) | ? | (Nurbaeva *et al.* 2015*a*) |
| ORAI3 | ↓ | ↑ | mRNA, function(?) | ? | (Nurbaeva *et al.* 2015*a*) |
| $Ca^{2+}$ extrusion molecules | | | | | |
| (i) $Ca^{2+}$ exchangers | | | | | |
| NCKX1 | ↑ | ↓ | mRNA | ? | (Hu *et al.* 2012) |
| NCKX2 | Same | Same | mRNA | ? | (Hu *et al.* 2012) |
| NCKX3 | ↑ | ↓ | mRNA | ? | (Hu *et al.* 2012) |
| NCKX4 | ↓ | ↑ | mRNA, protein expression | Yes | (Hu *et al.* 2012), (Parry *et al.* 2013), (Wang *et al.* 2014) |
| NCKX5 | ↓ | ↑ | mRNA | ? | (Hu *et al.* 2012) |
| NCKX6 | Same | Same | mRNA | ? | (Hu *et al.* 2012) |
| NCX | — | — | Function | — | (Okumura *et al.* 2010) |
| NCX1 | Same | Same | mRNA, protein, | ? | (Okumura *et al.* 2010; Lacruz *et al.* 2012*b*) |
| NCX2 | Not expressed | Not expressed | mRNA, protein | N/A | (Okumura *et al.* 2010) |
| NCX3 | ↑ | ↓ | mRNA, protein | ? | (Okumura *et al.* 2010; Lacruz *et al.* 2012*b*) |
| (ii) $Ca^{2+}$ pumps | | | | | |
| SERCA1 | Not expressed | Not expressed | mRNA | N/A | (Franklin *et al.* 2001), (Nurbaeva *et al.* 2015*a*) |
| SERCA2 | ↓ | ↑ | mRNA, protein, function | ? | (Franklin *et al.* 2001), (Nurbaeva *et al.* 2015*a*) |
| SERCA3 | Not expressed | Not expressed | mRNA | N/A | (Franklin *et al.* 2001), (Nurbaeva *et al.* 2015*a*) |
| PMCA | ? | ? | EM, protein | — | (Salama *et al.* 1987), (Borke *et al.* 1993) |
| PMCA1 | Expressed (?) | Expressed (?) | mRNA, protein | ? | (Borke *et al.* 1993) |
| PMCA2 | Not expressed | — | mRNA | N/A | (Borke *et al.* 1993) |
| PMCA3 | Not expressed | — | mRNA | N/A | (Borke *et al.* 1993) |
| PMCA4 | Expressed (?) | Expressed (?) | mRNA, protein | ? | (Borke *et al.* 1993) |

that in enamel cells, calmodulin expression was similar throughout the different stages of amelogenesis (Table 1) (Hubbard, 1995).

Calcineurin. Calcineurin is a $Ca^{2+}$/calmodulin-dependent protein phosphatase consisting of two subunits, the catalytic A (CnA) and the regulatory subunit B (CnB) of 60 kDa and 19 kDa, respectively (Ke & Huai, 2003). The latter contains four $Ca^{2+}$ binding EF-hand motifs and is bound to CnA in conditions of resting $Ca^{2+}$ levels (Furman & Norris, 2014). In mammals, there are three calcineurin isoforms (CnA$\alpha$, CnA$\beta$, CnA$\gamma$) and two CnB isoforms (CnB1 and CnB2), being highly conserved. Calcineurin is involved in many biological processes of which probably the best studied is its association with signal transduction pathways mediating T-cell activation thus making it a good candidate for drugs targeting immunosuppression (Crabtree, 2001). Calcineurin de-phosphorylates nuclear factor of activated T cells (NFAT), a process required for NFAT nuclear translocation and signalling activity (Crabtree, 2001).

In ameloblasts, CnA$\alpha$, CnA$\beta$ and CnB1 were identified by *in situ* hybridization during tooth differentiation at embryonic day 15 and by immunohistochemistry in secretory stage ameloblasts (Oshima & Watanabe, 2012). RA cells showed strong reactivity to CnA$\beta$ and CnB1 suggesting a role of calcineurin associated with the mineralization stage (Oshima & Watanabe, 2012).

**$Ca^{2+}$ binding proteins in ER lumen.** The total concentration of $Ca^{2+}$ within the ER lumen is approximately 1 mM, with free $Ca^{2+}$ in the range of approximately 200 $\mu$M and the remainder buffered via $Ca^{2+}$ binding proteins such as calreticulin, endoplasmin and ERp72 (Michalak & Opas, 2009). These proteins also play other roles within the cell being involved in protein folding and regulation of $Ca^{2+}$ release (Michalak & Opas, 2009).

Calreticulin. Calreticulin is a 46 kDa protein responsible for buffering up to 50% of ER $Ca^{2+}$ in non-excitable cells (Nakamura *et al.* 2001*a*, *b*). Structurally, calreticulin consists of three distinct domains N, P and C. N and P domains are implicated in chaperone function and the C domain is enriched in negatively charged amino acid residues responsible for its $Ca^{2+}$ buffering capabilities (Nakamura *et al.* 2001*b*). It binds $Ca^{2+}$ with high capacity and low affinity (Nakamura *et al.* 2001*b*). *In vivo* and *in vitro* models of calreticulin deficiency and over-expression have shown how tight control of protein folding and $Ca^{2+}$ homeostasis by calreticulin are necessary for proper function and development (Mesaeli *et al.* 1999; Nakamura *et al.* 2001*a*).

Endoplasmin. Endoplasmin is an ER resident protein of 94 kDa, with a $Ca^{2+}$ buffering role (Macer & Koch, 1988; Van *et al.* 1989). It binds $Ca^{2+}$ with high capacity (Macer & Koch, 1988; Van *et al.* 1989). Endoplasmin binds to ER luminal peptides and this binding is increased in the absence of $Ca^{2+}$, consistent with its role as a response to ER stress (Ying & Flatmark, 2006; Biswas *et al.* 2007).

ERp72. The primary function of ERp72 appears to be to act as a molecular chaperone (Nigam *et al.* 1994), where it isomerizes disulfide bonds (Rupp *et al.* 1994). Though it does bind $Ca^{2+}$, the chaperone activity of ERp72 is unaffected by $Ca^{2+}$ concentrations (Rupp *et al.* 1994) and overexpression of ERp72 does not increase ER $Ca^{2+}$ stores, suggesting that its protein folding activity is more important than its $Ca^{2+}$ binding function (Lievremont *et al.* 1997).

Detection of calreticulin and endoplasmin by Western blot showed that both were up-regulated in rat maturation ameloblasts (Table 1) (Hubbard, 1996), which might suggest that luminal ER [$Ca^{2+}$] is higher in maturation relative to secretory and requires compensation by these $Ca^{2+}$ buffering proteins. In contrast, ERp29 was up-regulated in rat secretory stage ameloblasts (Table 1), which might indicate the involvement of this protein in enamel specific protein production as ERp29 is linked to protein folding and chaperone activities (Hubbard *et al.* 2000).

Calnexin. Calnexin (CNX) is a 90 kDa type-I ER integral protein involved in protein folding following glucose removal of newly synthesized peptides (Ni & Lee, 2007). CNX contains luminal and cytosolic domains with the luminal domain (globular domain) containing a $Ca^{2+}$ binding site (Dudek *et al.* 2015). Abnormal CNX function has been associated with Alzheimer's disease (Ni & Lee, 2007). In enamel cells, CNX has been found in LS8 cells and in secretory and maturation stage ameloblasts (Nurbaeva *et al.* 2015*a*,*b*). In LS8 cells, its localization differed from that of the ER pump SERCA2 (see Pumps and exchangers section) being more limited in its distribution (Nurbaeva *et al.* 2015*b*). In maturation stage ameloblasts, CNX showed a similar distribution to both SERCA2 and STIM1 (Nurbaeva *et al.* 2015*a*).

## Pumps and exchangers

Tight regulation of $[Ca^{2+}]_i$ is important and necessary for all cells in order to avoid $Ca^{2+}$ toxicity. An important mechanism to maintain $[Ca^{2+}]_i$ within a normal range is through the activity of various pumps and exchangers which remove $Ca^{2+}$ from the cytoplasm such as ER/SR $Ca^{2+}$-ATPase (SERCA) or plasma membrane $Ca^{2+}$-ATPase (PMCA) pumps, or extruding $Ca^{2+}$ out of

the cell such as $K^+$-independent $Na^+/Ca^{2+}$ (NCX) and $K^+$-dependent $Na^+/Ca^{2+}$ exchangers (NCKX).

**Pumps.** Two main types of ATPase pumps mobilize $Ca^{2+}$ with distinct roles. SERCAs move cytosolic $Ca^{2+}$ into the ER lumen, whereas PMCAs pump cytosolic $Ca^{2+}$ out of the cell. SERCA maintains low $[Ca^{2+}]_i$ by exchanging protons for two $Ca^{2+}$ per ATP hydrolysed, and PMCA transports one $Ca^{2+}$ out of the cell per ATP hydrolysed. PMCA and SERCA pumps have lower transport rates but high $Ca^{2+}$ affinity and thus they can respond only to modest elevations in $[Ca^{2+}]_i$.

SERCA. SERCA pumps are encoded by three different genes, *ATP2A1–3*, each encoding several protein isoforms. *ATP2A1* gene encodes SERCA1a and b isoforms, *ATP2A2* gene encodes SERCA2a, b and c, and *ATP2A3* gene encodes six isoforms of SERCA3a–f, respectively (Hovnanian, 2007; Zarain-Herzberg *et al.* 2014). SERCA expression shows distinct patterns of tissue distribution (Missiaen *et al.* 2000; Shull *et al.* 2003; Periasamy & Kalyanasundaram, 2007) so that mutations in each SERCA isoform differentially affect organ system function resulting in a number of diseases such as respiratory failure, Brody myopathy, dominant skin disease and Darier disease (Foggia & Hovnanian, 2004; Hovnanian, 2004, 2007).

SERCA in ameloblasts. SERCA was first identified in enamel organ cell extracts by RT-PCR, cDNA cloning, Northern analysis and immunoblotting all of which suggested that SERCA2b was the main isoform expressed by the enamel organ (Franklin *et al.* 2001). No *SERCA2a* transcript has been detected in enamel cells (Franklin *et al.* 2001). It was also reported that SERCA2b expression increased at the maturation stage (Table 1) (Franklin *et al.* 2001). We have since identified the *ATP2A1–3* genes in rat secretory and maturation ameloblasts as well as in LS8 cells by RT-PCR (Nurbaeva *et al.* 2015*a*,*b*) and confirmed that SERCA2 was the predominant SERCA at both the transcript and protein levels (Nurbaeva *et al.* 2015*a*). SERCA2 localization in maturation stage ameloblasts showed wide cytosolic distribution overlapping with that of the ER protein CNX (Nurbaeva *et al.* 2015*a*). Thapsigargin is the most widely used SERCA inhibitor, which results in cytosolic $Ca^{2+}$ increases as SERCA no longer pumps $Ca^{2+}$ into the ER lumen leading to a passive depletion of $Ca^{2+}$ stores through poorly understood $Ca^{2+}$ leaks in the ER. Rat secretory and maturation ameloblasts as well as LS8 cells showed $[Ca^{2+}]_i$ increase following stimulation with thapsigargin in the absence of extracellular $Ca^{2+}$ due to $Ca^{2+}$ release from the ER stores, providing direct evidence of a functional SERCA (Nurbaeva *et al.* 2015*a*,*b*).

PMCAs. PMCAs (~140 kDa) include a subfamily of the P-type ATPase superfamily characterized by the formation of a phosphorylated enzyme during ATP hydrolysis (Strehler, 2015). Four distinct PMCA isoforms (PMCA 1–4) are commonly identified in mammals encoded by distinct genes (*ATP2B1*, *ATB2B2*, *ATP2B3* and *ATP2B4* in humans) located on separate chromosomes (Strehler *et al.* 2007). PMCA1 and PMCA4 are ubiquitous, with the former having a housekeeping role being expressed during early development (Brini & Carafoli, 2011). PMCA2 is widely distributed in all areas of the brain and is also found in the organ of Corti in the middle ear (Furuta *et al.* 1998; Dumont *et al.* 2001). PMCA2 also regulates $Ca^{2+}$ homeostasis in the retina. PMCA3 is highly expressed in skeletal muscles (Lopreiato *et al.* 2014; Strehler, 2015). PMCA2 mutations result in deafness and equilibrium disturbances as well as impaired retinal function (Duncan *et al.* 2006). Overexpression of PMCA2 increased the $Ca^{2+}$ content in milk (Reinhardt *et al.* 2004). PMCA3 has never been successfully knocked out suggesting an essential function of this isoform. Mutations in *ATP2B3* result in male sterility (Schuh *et al.* 2004).

PMCA in ameloblasts. Expression and plasma membrane association of PMCA was shown in both secretory and maturation stage ameloblasts in the rat incisors using enzyme cytochemistry, immunochemical techniques and mRNA expression (Inage & Weinstock, 1979; Crenshaw & Takano, 1982; Sasaki & Garant, 1986; Takano *et al.* 1986; Salama *et al.* 1987, 1989; Sasaki *et al.* 1987; Eisenmann *et al.* 1990; Borke *et al.* 1993, 1995; Takano, 1995; Zaki *et al.* 1996). mRNA transcript analysis showed that *PMCA-1* and *PMCA-4* are the predominant isoforms in human and rat ameloblasts (Table 1) (Borke *et al.* 1995; Zaki *et al.* 1996), as we have also identified in our lab (unpublished data). However, there seems to be a lack of consensus concerning expression pattern differences in stages as well as disagreement on its cellular localization. One study reported that PMCA expression in the secretory stage was associated with the entire PM (Salama *et al.* 1987). During early and late maturation, RA showed high PMCA intensity along the distal ruffled border (Salama *et al.* 1987). During early and late maturation, SA cells showed substantial PMCA expression along the lateral and proximal surfaces but not at the distal plasma membrane (Salama *et al.* 1987). A subsequent study by Borke and colleagues investigated the expression of PMCA epitopes in rat incisor formation and mineralization using a monoclonal antibody and found that PMCA epitopes are present in all stages of amelogenesis and the location and intensity of staining of PMCA epitopes also varies with the progress of tissue mineralization (Borke *et al.* 1993). Early maturation ameloblasts often exhibited greater staining intensity than secretory ameloblasts (Borke *et al.* 1993). More recently,

detection and distribution of PMCA in ameloblasts used a specific monoclonal antibody and immunogold cytochemistry analysed by electron microscopy (Zaki *et al.* 1996). The highest concentration of gold particles were observed in the distal membranes of early-maturation ameloblasts relative to late-maturation and secretory stage cells (Zaki *et al.* 1996).

In addition, PMCA activity was sensitive to the calmodulin inhibitor trifluoperazine. Trifluoperazine yielded partial systemic inhibition of enamel mineralization during the secretory stage but had no effect during the maturation stage (Sasaki *et al.* 1987). It also induced morphological changes in secretory stage ameloblasts. Enamel crystallites of trifluoperazine-injected rats appeared to be less electron-dense than those from controls. In part, some of the discrepancies in the results described above have been attributed to a variety of chemicals and processing of tissues used by different laboratories (Takano, 1995). It should also be noted that $Ca^{2+}$-ATPases have also been described in mitochondria and Golgi complex in ameloblasts (Sasaki *et al.* 1997) and it has been considered that in Golgi saccules, $Ca^{2+}$ might be condensed into secretory granules (Eisenmann *et al.* 1982).

## Exchangers

Besides SERCA and PMCA, there are other $Ca^{2+}$ clearance mechanisms in cells, such as NCX and NCKX. An important difference between these exchangers and the pumps is that the turnover rate of exchangers is orders of magnitude higher than that of ATP-driven $Ca^{2+}$ pumps (Herchuelz *et al.* 2007).

**NCX and NCKX.** Three NCX and six NCKX isoforms have been identified by molecular cloning in mammalian cells (Herchuelz *et al.* 2007; Lytton, 2007; Visser & Lytton, 2007; Visser *et al.* 2007). NCX isoforms remove a single $Ca^{2+}$ in exchange for three $Na^+$ (Yu & Choi, 1997), whereas NCKX isoforms co-transport one $K^+$ and one $Ca^{2+}$ in exchange for four $Na^+$ (Lytton, 2007). NCKX and NCX are bidirectional electronic transporters depending on the prevailing electrochemical driving forces, i.e. the $Na^+$ and $Ca^{2+}$ concentrations and the membrane potential, exchangers accomplishing $Ca^{2+}$ entry or $Ca^{2+}$ exit. NCKX1 is predominantly expressed on rod photo-receptors and platelets, NCKX4 are found in the brain, and NCKX3 and 4 isoforms are predominant in aorta. NCKX2 is expressed in retinal cone photo-receptors and retinal ganglion cells, NCKX3 is expressed in uterus and intestine, and NCKX4 is in lung thymus (Schnetkamp, 2004). NCX and NCKX isoforms are expressed in human lung macrophages, dendritic cells and blood monocytes (Staiano *et al.* 2009; Shumilina *et al.* 2010; Heise *et al.* 2011). NCX and NCKX are encoded by the *SLC8A* and *SLC24A4* genes, respectively. NCX1, NCX3 and NCKX1–6 isoforms have been demonstrated to be expressed on rat ameloblasts albeit showing different expression levels (Okumura *et al.* 2010; Hu *et al.* 2012).

**NCKX and NCX in ameloblasts.** The expression of $Na^+/Ca^{2+}$ exchanger family NCX in enamel cells was first reported by Okumura and colleagues (Okumura *et al.* 2010). NCX1 and NCX3 but not NCX2 were found in these cells both showing an apical or apico-lateral distribution. NCX1 also showed limited basal reactivity. NCX involvement in $Ca^{2+}$ transport was further demonstrated by electrophysiological analysis of whole cell recordings as well as the use of pharmacological inhibitors of NCX. These data are unique in that they represent the only study to date, to the best of our knowledge, where ameloblasts have been used to perform electrophysiological experiments. However, a caveat in the experimental design is that the cells used derived from 7-day-old rats, which makes it unlikely that the data obtained fully represented the maturation stage. Indeed, despite the use of alkaline phosphatase as a maturation stage marker, the histological analysis reveals only moderate amounts of tissue thickness formed in enamel and dentine, and cells labelled as maturation stage ameloblasts show the presence of stratum intermedium cells (Okumura *et al.* 2010), all of which is inconsistent with maturation. Despite this, the data implicate NCX in $Ca^{2+}$ transport in at least one type of ameloblast (secretory). This is an important advance in deciphering the molecular tools available to ameloblasts to export an excess of intracellular $Ca^{2+}$ to the enamel compartment. Our assessment of NCX1 and NCX3 expression by RT-PCR showed that neither of these genes increased expression during the maturation stage and hence we suggested the possibility of additional exchangers would be implicated in $Ca^{2+}$ extrusion at this critical stage (Lacruz *et al.* 2012*a*). It might be the case that $Ca^{2+}$-ATPases combined with NCX activity provide secretory ameloblasts with the necessary mechanisms to enable sufficient $Ca^{2+}$ to be extruded through the apical pole to ensure elongation of the thin enamel crystals.

Soon after reporting on NCX, our genome wide study comparing secretory *vs.* maturation stage enamel organs, identified the expression of NCKX4, a $Na^+/K^+/Ca^{2+}$ exchanger, as being markedly up-regulated in maturation (Lacruz *et al.* 2012*a*) and investigated the expression of the NCKX family in enamel cells. We found that all members of the NCKX family were expressed at varying levels in both secretory and maturation stages but that NCKX4 dominated the expression profile during the maturation stage, thus suggesting an important role in enamel formation (Hu *et al.* 2012). Moreover, on evidence from peroxidase staining, secretory stage ameloblasts did not show reactivity to NCKX4 (Hu *et al.* 2012). Maturation

stage ameloblasts showed strong reactivity at the apical end (Hu *et al.* 2012). Immunofluorescence studies shown here confirmed the predominant expression of NCKX4 at the apical pole but only in RA cells. SA cells showed a different staining pattern being more diffusely distributed through the cell (Fig. 4) highlighting potential differences in $Ca^{2+}$ extrusion between the two maturation stage cell types. The localization of NCKX4 to the apical end of the cell places it in the right side of the cells to be involved in $Ca^{2+}$ extrusion (Fig. 4). This finding was supported by subsequent reports characterizing severe enamel defects in patients and in mouse models with mutations to *SLC24A4* (the gene encoding for NCKX4) (Parry *et al.* 2013; Wang *et al.* 2014). Four mutations in *SLC24A4* gene have been identified resulting in amelogenesis imperfecta such as Arg339Ter (C.1015C>T), Ser499Cys (C.1495A>T) (Parry *et al.* 2013), Ala146Val (Wang *et al.* 2014) and Leu436Arg (G.165151 T>G; C.1317 T>G) (Herzog *et al.* 2015) (Fig. 4*A*). These reports described the enamel phenotype in human patients as displaying a yellow-brown discolouration having increased opacity but with normal enamel volume and crown morphology, although affected teeth seemed susceptible to premature enamel loss (Parry *et al.* 2013). However, in mice, enamel was only present at the base of the tooth (cervical margin) and was missing from the remainder of the tooth (Parry *et al.* 2013). Thus, the role of NCKX4 in $Ca^{2+}$ extrusion in ameloblasts has been strengthened although it would be desirable to obtain electrophysiological data to confirm this.

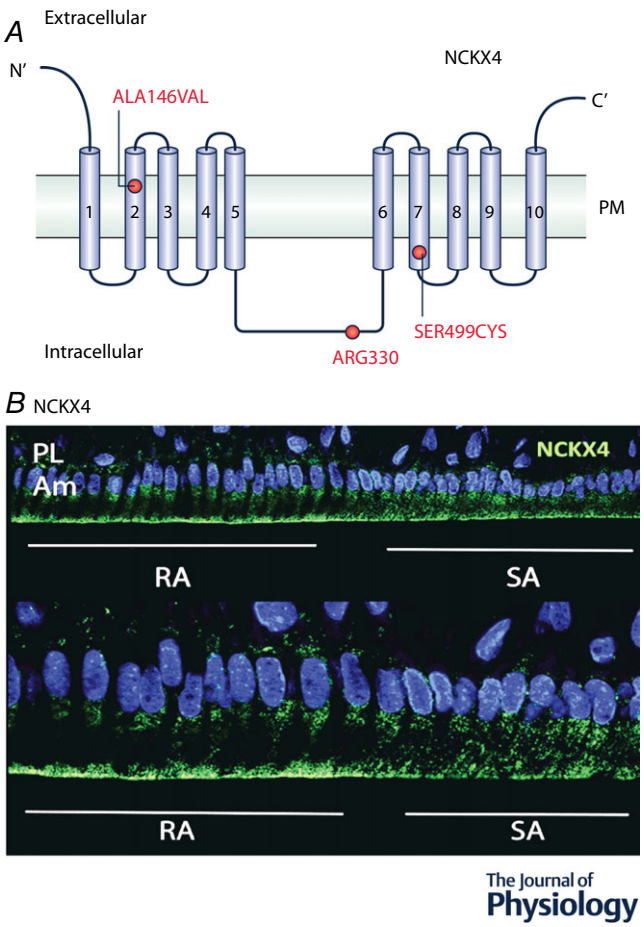


**Figure 4. NCKX4 mutations and localization in ameloblasts**
*A*, schematic representation of NCKX4 protein structure showing the 10 transmembrane domains and corresponding loops. The red dots indicate the mutations at each domain that have been associated with enamel deficiencies. PM = plasma membrane. *B*, NCKX4 localization in rat ameloblasts. Immunofluorescence microscopy analysis of NCKX4 localization in RA and SA ameloblasts. In RA cells, NCKX4 is largely localized at the apical (distal) pole of the cell. In SA cells, NCKX4 distribution becomes more diffused suggesting a principal role for this protein in $Ca^{2+}$ extrusion during the RA stage. DAPI is shown in blue. RA = ruffled-ameloblasts, SA = smooth-ameloblasts, Am = ameloblasts, PL = papillary layer.

**Voltage independent and voltage dependent $Ca^{2+}$ channels.** Ameloblast cells are considered non-excitable cells and hence it is interesting to find that L-type voltage-gated ($Ca_v1.2$) channels are associated with abnormal enamel (Papineau & Wilson, 2014). Mutations in the gene encoding for $Ca_v1.2$ (*CACNA1C*) result in hypoplastic amelogenesis imperfecta (Papineau & Wilson, 2014). However, there is at present no available data on *CACNA1C* expression in enamel cells. In contrast, voltage-independent $K^+/Ca^{2+}$ activated channel KCNN4, activated by intracellular $Ca^{2+}$, was reported by us as being up-regulated during the maturation stage in a genome wide study (Lacruz *et al.* 2012*a*), although at present we have no other expression data and no mutations to this gene have been linked with abnormal enamel.

## Developing a model for $Ca^{2+}$ signalling and transport in enamel

A summary of $Ca^{2+}$ associated proteins thus far identified in enamel cells is shown in Fig. 5. It is now well accepted that the transport of ions to form and mature enamel crystals follows a basal to apical route across the ameloblast cell barrier. The principal mode of transport appears to be the transcellular route but the contribution of a paracellular passage of ions during the RA to SA cycles cannot be discounted. $Ca^{2+}$ influx has been regarded as a passive event and was tightly coupled to a concentration gradient difference between the low cytosolic [$Ca^{2+}$] and the much greater extracellular [$Ca^{2+}$]. The rise in [$Ca^{2+}$]$_i$ via passive influx occurred near the basolateral pole partly modulated by the hypothetical action of unknown exchangers located midway along the cell body that extruded an excess of $Ca^{2+}$ (e.g. Bawden, 1989; Takano, 1995; Hubbard, 2000). A key point in these models was the consideration that CaBP enabled intracellular transport across the cell, which also benefited from a lower [$Ca^{2+}$]

at the apical pole so that CaBP acted as $Ca^{2+}$ shuttles in the basal-to-apical direction without raising $[Ca^{2+}]_i$ to toxic levels. As far as the extrusion of $Ca^{2+}$ into the enamel was concerned, PMCAs were largely involved in this process (Bawden, 1989).

However, Hubbard reworked this model invoking not a CaBP cytosolic transport route but an organellar-based transfer of $Ca^{2+}$ across the cell. In fact, Hubbard (Hubbard, 2000) considered the possibility that SOCE might be involved in $Ca^{2+}$ transport although evidence for this was limited. More recently, Feske also considered the potential role of SOCE in $Ca^{2+}$ transport in enamel epithelium (Feske, 2009). Indeed, our recent data strongly support this model for $Ca^{2+}$entry (Nurbaeva *et al.* 2015*a*,*b*). Our functional studies showing markedly decreased $Ca^{2+}$ entry in enamel organ cells pre-treated with the CRAC inhibitor Synta 66 demonstrated that this path is important for $Ca^{2+}$ influx into these cells. Strong supporting evidence is found in the severe enamel phenotypes in patients with mutations to either *STIM1* or *ORAI1*. Clearly, CRAC channel function is tied to enamel development and mineralization. A caveat, however, is that to fully understand the effects of SOCE deficiency in enamel formation, the dentition of patients with *STIM1/ORAI1* mutations and that of animal models should be analysed in detail. Unfortunately, *Stim1*-deficient animals do not survive well after birth (Oh-Hora *et al.* 2008) and to date no comprehensive analysis of the enamel of teeth derived from human patients has been performed. Despite this important caveat, $Ca^{2+}$ entry in enamel cells can now be largely considered as being modulated by CRAC channels.

Intracellular $Ca^{2+}$ transport across the cell remains the biggest unknown. Hubbard's group had discounted the importance of CaBP as no enamel defects had been identified in mice lacking a number of abundantly expressed CaBP in enamel cells (Hubbard *et al.* 2011). This might direct a focus towards an organellar route. The ER forms a continuous tunnel-like network of

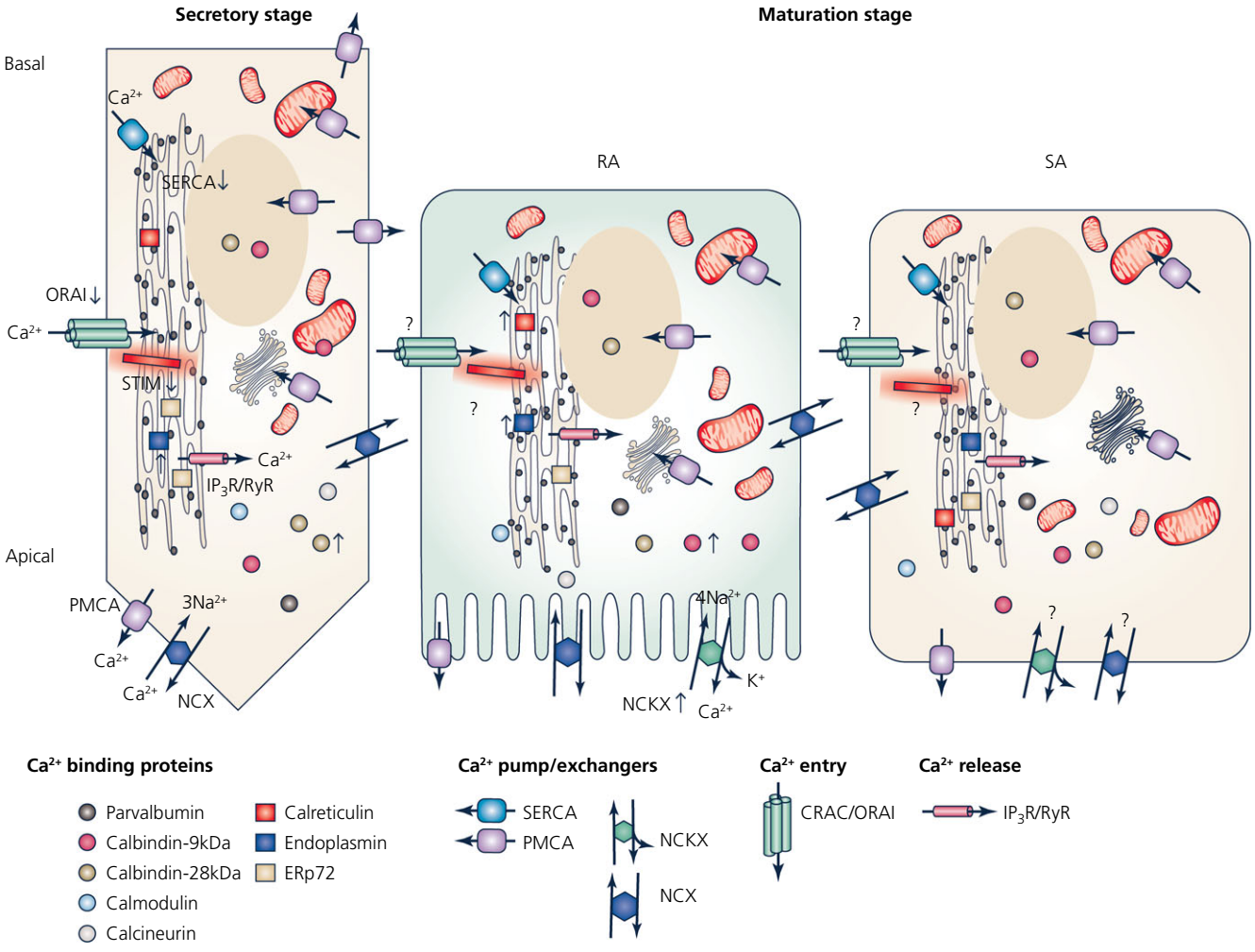


**Figure 5. $Ca^{2+}$ toolkit in ameloblasts**
Schematic representation of $Ca^{2+}$ handling proteins that have been reported to date in secretory and maturation stage ameloblasts. See text for details. RA = ruffled-ameloblasts, SA = smooth-ameloblasts.

structures transporting $Ca^{2+}$ within this network in acinar and brain cells (Mogami *et al.* 1997; Park *et al.* 2000; Levine & Rabouille, 2005), but this is yet to be tested in ameloblasts, although it remains an attractive possibility. Within the ER lumen, enamel cells express calreticulin, endoplasmin, ERp72 and calnexin, which contribute to luminal $Ca^{2+}$ buffering (Fig. 5). The ER membrane bound SERCA pumps are indeed expressed and have an active role in enamel cells as evidenced by inhibiting its function with thapsigargin. Thus, SERCA2 modulates cytosolic $Ca^{2+}$ buffering in enamel cells although SERCA2 mutations do not seem to affect enamel despite its relative abundance. One could speculate that in such cases other SERCA types could compensate enabling some level of $Ca^{2+}$ sequestration into the ER. Other cytosolic and ER luminal $Ca^{2+}$ buffers are known to be expressed in enamel cells (e.g. parvalbumins, calbindins, calretinin, calmodulin, calcineurin) and can in principle play roles in modulating $[Ca^{2+}]$ but these roles might also be either limited or stimulate compensatory mechanisms as no mutations to any of these proteins result in enamel defects (Table 1).

Alternative models for the safe intracellular transport of $Ca^{2+}$ have been suggested. One such possibility is the packing of $Ca^{2+}$ into vesicles within the Golgi saccules which are then exocytosed at the apical pole (Eisenmann *et al.* 1982). In addition, mitochondrial mobility coupled with strong GBHA signals identified in mitochondria in secretory and maturation ameloblasts, suggested a potential role for these $Ca^{2+}$ storage organelles in transporting $Ca^{2+}$ towards the apical pole (Takano *et al.* 1989). Yet it is somewhat surprising that GBHA did not identify signals emanating from the principal intracellular stores, namely the ER. Regardless, the role of mitochondria in intracellular $Ca^{2+}$ transport remains to be investigated.

The extrusion mechanisms appear also to have been decoded to some extent. PMCAs are expressed in enamel cells as reported by a number of studies so they probably play a $Ca^{2+}$ extrusion role. A lower expression of PMCAs in the secretory stage relative to maturation is in keeping with the notion that $Ca^{2+}$ requirements increase during the latter stage. Furthermore, PMCA expression in the distal ruffled border of RA cells has prompted some investigators to suggest that this cellular localization might enable proton removal from the enamel zone (Hubbard, 2000). Protons are released during enamel crystal formation which could impact local pH (Lacruz *et al.* 2012*b*) so PMCAs would thus contribute to this clearing process while pumping $Ca^{2+}$ outside the cell. PMCAs require ATP for transport and thus have more limited clearing dynamics when compared with NCX and NCKX exchangers as these are ATP independent (Fig. 5).

Neither a genome wide analysis comparing secretory and maturation stage enamel organs nor mRNA screening suggests that NCXs are up-regulated in maturation which might point to a more housekeeping role for this exchanger family. Importantly no mutations to NCX have been linked to disruptions of the enamel whereas a number of mutations in the NCKX4 encoding gene (*SLC24A4*) result in phenotypes resembling amelogenesis imperfecta (Parry *et al.* 2013; Wang *et al.* 2014). This points to a more substantial role for NCKX4 despite both having similar $Ca^{2+}$ kinetics exchanging only one $Ca^{2+}$.

Thus, a current conceptual model for gated $Ca^{2+}$ transport has the following characteristics (see also Fig. 5): $Ca^{2+}$ uptake is dominated by the function of CRAC channels, which are probably activated in response to PLC mediated $IP_3R$ $Ca^{2+}$ release from the ER in response to a yet unknown agonist. The cytosolic rise of $[Ca^{2+}]$ via ORAI1 opening is compensated by common cytosolic buffers. SERCA2 replenishes luminal $Ca^{2+}$, which is bound then to a number of luminal buffers. In secretory stage ameloblasts, $Ca^{2+}$ entry might be mediated by STIM2 as its better known homologue STIM1 is nearly absent at this stage. ORAI1 is expressed at low levels in secretory ameloblasts but is clearly present. Efflux of $Ca^{2+}$ in secretory cells might be mediated by NCX and PMCA extruding $Ca^{2+}$ principally across the apical pole of the cell. The reported expression of PMCAs in the lateral cell membrane of secretory ameloblasts is intriguing as the intercellular space is extremely limited and the passage of ions to the enamel is hampered by the presence of tight junctions apically. Thus, lateral efflux of $Ca^{2+}$ may be used as a mechanism to remove a small amount of $Ca^{2+}$ which might slowly diffuse basally to be recycled or it can slowly leak across the tight junctions distally. In maturation stage ameloblasts we envision a NCKX4 dominated $Ca^{2+}$ clearing function with PMCA playing a lesser role. More specifically, this pattern might be more closely associated with RA cells, whereas in SA cells this is less clear. In this regard, the important morphological changes in organellar distribution that are seen in the latter stage might have an impact in $Ca^{2+}$ transport but this is far from clear. Regardless, these data combined enable us to construct a working model depicting the main molecular components enabling $Ca^{2+}$ transport in enamel epithelium (Fig. 5).

## Conclusion

Despite recent efforts in deciphering aspects of $Ca^{2+}$ dynamics in enamel formation and mineralization, many unknowns still remain. Enamel cells are now recognized as a cell model expressing CRAC channels and show many similar components of the $Ca^{2+}$ dynamic toolkit found in many other cell systems opening expansive future research avenues. For example we have previously suggested that increased levels of $[Ca^{2+}]_i$ via CRAC channels have a direct effect in the expression of enamel genes although how

this process is modulated remains unknown. This direct signalling mechanism can be a potent mediator of many cellular responses leading to protein synthesis and export in enamel cells as it is recognized in other cell systems. ER $Ca^{2+}$ release in itself does not appear to affect the response of enamel genes suggesting that a more sustained or an elevated rise in $[Ca^{2+}]_i$ is required. Considering that CRAC channel mediated $Ca^{2+}$ influx is necessary for the activation of NFAT known to have a role in vertebrate development and bone homeostasis, its potential role in enamel development given that NFATs are expressed in enamel cells (our unpublished data) might be of interest. A better understanding of $Ca^{2+}$ dynamics in the complex system represented by enamel cells should thus have an immediate appeal to those interested not only in enamel biology, but also to the larger community involved in $Ca^{2+}$ signal transduction and $Ca^{2+}$ regulation at large.

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## Additional information

### Competing interests

S.F. is a cofounder of Calcimedica; the other coauthors declare no conflict of interest.

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