Published in final edited form as:

Matrix Biol. 2016 ; 52-54: 234–245. doi:10.1016/j.matbio.2015.11.004.

V-type ATPase Proton Pump Expression During Enamel Formation

Juni Sarkar¹, Xin Wen¹, Emil J. Simanian¹, and Michael L. Paine^{1,*}

¹ Center for Craniofacial Molecular Biology, Herman Ostrow School of Dentistry of USC, University of Southern California, Los Angeles, 90033, USA

Abstract

Several diseases such as proximal and distal renal tubular acidosis and osteoporosis are related to intracellular pH dysregulation resulting from mutations in genes coding for ion channels, including proteins comprising the proton-pumping V-type ATPase. V-type ATPase is a multi-subunit protein complex expressed in enamel forming cells. V-type ATPase plays a key role in enamel development, specifically lysosomal acidification, yet our understanding of the relationship between the endocytotic activities and dental health and disease is limited. The objective of this study is to better understand the ameloblast-associated pH regulatory networks essential for amelogenesis. Quantitative RT-PCR was performed on tissues from secretory-stage and maturation-stage enamel organs to determine which of the V-type ATPase subunits are most highly upregulated during maturation-stage amelogenesis: a time when ameloblast endocytotic activity is highest. Western blot analyses, using specific antibodies to four of the V-type ATPase subunits (Atp6v0d2, Atp6v1b2, Atp6v1c1 and Atp6v1e1), were then applied to validate much of the qPCR data. Immunohistochemistry using these same four antibodies was also performed to identify the spatiotemporal expression profiles of individual V-type ATPase subunits. Our data show that cytoplasmic V-type ATPase is significantly upregulated in enamel organ cells during maturation-stage when compared to secretory-stage. These data likely relate to the higher endocytotic activities, and the greater need for lysosomal acidification, during maturation-stage amelogenesis. It is also apparent from our immunolocalization data, using antibodies against two of the V-type ATPase subunits (Atp6v1c1 and Atp6v1e1), that significant expression is seen at the apical membrane of maturation-stage ameloblasts. Others have also identified this V-type ATPase expression profile at the apical membrane of maturation ameloblasts. Collectively, these data better define the expression and role of the V-type ATPase proton pump in the enamel organ during amelogenesis.

*Corresponding author, Michael Paine: 2250 Alcazar St, CSA 103, Los Angeles, CA 90033, Phone: (323) 442-1728, Fax: (323) 442-2981, ; Email: paine@usc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Keywords

V-type ATPase; amelogenesis; pH regulation and ATP6 subunits

INTRODUCTION

pH regulation modulates the crystal growth in the enamel microenvironment critical for proper enamel biomineralization [1,2]. Enamel crystallite growth and extracellular proteinase activity (to remove extracellular enamel matrix organic materials) are pH-dependent and require the interplay of a number of ion transporters [3,4,5,6,7,8,9]. During enamel formation, ameloblast-associated crystallite growth and endocytosis are most prominent during the later stages of amelogenesis (i.e. maturation-stage) [10,11]. Protons acidify the microenvironment of the ameloblasts, and require tight pH regulation to prevent any disruption to guided crystal growth [1,2,12]. V-type ATPase (also known as Vacuolar ATPase or H⁺ATPase) is a protein complex associated with the endosomes and lysosomes [13]. V-type ATPase plays a critical role in maintaining cellular pH homeostasis through a proton pumping activity [6,14,15]. The internal environment of the lysosome must be maintained at a pH ~ 4.0-5.0 to be functional, thus making extreme demands of a constant proton (H⁺) influx and chloride efflux [16,17]. The passage of protons into the lysosome lumen through the V-type ATPase complex is driven by ATP hydrolysis, with the resulting voltage difference across the lysosomal membrane requiring additional ion transport [18].

The V-type ATPase is composed of two joined multi-protein complexes: cytoplasmic “ATP-hydrolyzing” V₁ complex and the membrane-bound “proton pump” V₀ complex [19]. The V₁ domain subunits are typically designated as A-H isoforms (**Table 1**). V₀ domain subunits are also designated as the A-E isoforms (Table 1). Further, multiple genes code for individual subunits such that there are currently twenty-two known genes (see Table 1) coding for thirteen V-type ATPase subunits. Additionally, the generation of multiple spliced variants is a feature of many of these V-type ATPase genes [20].

In a genome wide microarray analysis of the enamel organ comparing the secretory and maturation stages of enamel formation, we have previously shown that some of the V-type ATPase subunits were significantly upregulated during late-stage amelogenesis [10,21,22]. A connection between V-type ATPase function and enamel formation is evident in mouse, where a 72kDa subunit of the V-type ATPase is seen localized primarily at the ruffled border in ruffle-ended ameloblasts (RA), with lesser expression observed at the apical pole of smooth-ended ameloblasts (SA) [23]. A more recent study by Josephsen et al. [6], using an antibody to the Atp6v0a1 subunit of V-type ATPase, also suggests that the V-type ATPase proton pump is most highly expressed at the apical pole of ruffle-ended ameloblasts, and may be involved with the active export of protons during enamel maturation [6,24]. The goal of our current study is to better define the expression levels of individual V-type ATPase subunits in the enamel organ during the various stages of amelogenesis, and to describe the spatiotemporal expression profiles of the V-type ATPase subunits in lysosomal, endosomal and plasma membranes.

RESULTS

Gene expression of V-type ATPase subunits

Previously, using DNA array analysis, we identified that endocytotic-related activities were associated with the maturation-stage of amelogenesis [10,21,22]. The array data [21,22], and subsequent immunolocalization data [10], show that a number of the subunits of V-type ATPase are present in enamel organ cells, and that the expression levels of many of these individual subunits increase significantly in the maturation-stage amelogenesis (when compared to secretory-stage amelogenesis) thus affirming further investigation. Here, all 22 V-type ATPase genes (Table 1) were examined to determine their mRNA expression profiles in the enamel organ during amelogenesis. Quantitative PCR (qPCR) on secretory-stage and maturation-stage rat enamel organ cells was used to better define V-type ATPase activities in amelogenesis. All *in vivo*, rat-derived data were normalized to *Actb* gene transcript levels, and *Enam* and *Odam* transcript served as additional controls [10,21]. As expected, *Enam* mRNA was significantly downregulated, and *Odam* gene transcripts were significantly upregulated, during enamel maturation [5]. A graphical representation of these qPCR results is shown in **Figure 1**. These qPCR data suggest that many of the V_0 subunits (Atp6v0a1, Atp6v0a3, Atp6v0d2, Atp6v0e1 and Atp6v0e2) are expressed at significantly higher mRNA levels at maturation-stage, when compared to secretory-stage (Figure 1). In the V_1 complex we also found that many subunit transcripts (Atp6v1b1, Atp6v1c1, Atp6v1e1, Atp6v1f, Atp6v1g1 and Atp6v1h) are expressed at significantly higher levels in maturation-stage enamel organ cells when compared to secretory-stage. Of note is that 5 of the 22 V-type ATPase genes/subunits (Atp6v0e1, Atp6v1c1, Atp6v1e1, Atp6v1f and Atp6v1h) show a > 10 fold higher expression at maturation-stage when compared to secretory-stage (Figure 1).

V-type ATPase protein expression

Western blot analysis was performed on selected V-type ATPase subunits (note that the selection was based on antibody availability with a bias to subunits where mRNA levels significantly increased during enamel maturation). Western blot analysis was performed to identify Atp6v0d2, Atp6v1b2, Atp6v1c1 and Atp6v1e1 in the protein lysate from rat enamel organ cells (**Figure 2**). Gapdh was used as a normalizing control. For the most part, the selected protein expression profiles reflected the levels observed for the mRNA expression profile, with *Amelx* and *Odam* expression serving as controls where *Amelx* expression is highest during secretory-stage and *Odam* expression is highest during maturation-stage. Consistent with the data observed in real-time PCR, Atp6v0d2, Atp6v1c1 and Atp6v1e1 were expressed in enamel organ cells and their expression clearly increased from secretory-stage to maturation stage (Figure 2). Although mRNA levels of Atp6v1b2 were not significantly different in secretory and maturation stages (Figure 1), our Western data shows higher protein levels in the maturation-stage (Figure 2).

Immunolocalization of selected V-type ATPase subunits during amelogenesis

Immunohistochemistry was performed to examine the *in vivo* expression profiles in the different enamel organ cell populations (**Figure 3-6**). The data presented here show the expression pattern of the above subunits Atp6v0d2, Atp6v1b2, Atp6v1c1 and Atp6v1e1 from secretory- to late-maturation stages during amelogenesis (Figure 3A, 4A, 5A and 6A).

Atp6v0d2 illustrates weak expression in secretory-stage ameloblasts (Figure 3 a1, a2), which increases considerably in maturation-stage ameloblasts. During maturation-stage amelogenesis there is a diffused expression of Atp6v0d2 in both the ameloblasts and the papillary layer cells (Figure 3 b1, b2). Signal is slightly elevated at the apical (distal) end of the ameloblasts (Figure 3 b1, b2). This data is consistent with our previously published data where we observed that immunolocalization of Atp6v0d2 was localized in maturation-stage ameloblasts and also the surrounding papillary layer cells [10]. Atp6v1b2 did not show any detectable staining at secretory-stage (Figure 4 a1, a2) while in the maturation-stage there was prominent diffused expression in both the ameloblasts and papillary layer cells (Figure 4 b1, b2). Atp6v1c1 and Atp6v1e1 each showed similar expression pattern of the enamel organ (Figure 5 and 6 respectively). Expression patterns of Atp6v1c1 and Atp6v1e1 revealed weak expression noted in the entire enamel epithelial organ during the secretory-stage (Figure 5 a1, a2 and Figure 6 a1, a2 respectively). The patterns also revealed a noticeable increase in both ameloblasts and papillary layer cells in early maturation-stage (Figures 5A and 6A respectively). In the late-maturation stage (Figure 5 b1, b2 and Figure 6 b1, b2), the strongest expression levels for the Atp6v1c1 and Atp6v1e1 subunits were in ameloblasts. Noticeably, during maturation stage, distinct staining pattern was observed for both Atp6v1c1 and Atp6v1e1 in both the ruffle-ended cells (RA) and smooth-ended (SA) ameloblast morphologies (Figure 5 b2 and Figure 6 b2). For both Atp6v1c1 and Atp6v1e1, RA cells demonstrated a diffused cytoplasmic staining with the strongest protein expression at the apical pole (best appreciated in Figure 5 b2 and Figure 6 b2).

In summary, the expression of all the four V-type ATPase subunits tested in ameloblasts revealed a gradual increase from the secretory-stage to early and late maturation-stages. Within secretory-stage ameloblasts the localization of V-type ATPase was primarily cytoplasmic, and as the ameloblasts became more mature the cytoplasmic staining became more intense. Clearly, there was noticeable expression at the apical plasma membrane of maturation-stage ameloblasts. Notable expression was also observed in the papillary layer cells. This data is consistent with the results of Western blotting shown in Figure 2.

DISCUSSION

The role of lysosomes in both intra and extracellular degradative processes during amelogenesis has been identified and discussed as early as 1969 [11]. In 1988 Sasaki et al. showed that lysosomes localize in vesicles of the ameloblasts, stratum intermedium cells and papillary layer cells [25]. An integral component of the lysosome is the proton-pumping V-type ATPase [13]. Recently, researchers have given greater attention to understanding the structural and functional basis of V-type ATPase in amelogenesis [4,6,24,26]. Josephsen et al. present some profound observations on the localization of V-type ATPase in the enamel organ. Using an antibody to Atp6v0a1, they discovered that this proton pump is highly expressed at early maturation-stage in ruffle-ended ameloblasts (RA) at the ruffled border, and that the proton pump is more internalized (cytoplasmic) in smooth-ended ameloblasts (SA) [6]. It was suggested that based on this unique expression profile for V-type ATPase in ameloblasts, in addition to a role in endosomal and lysosomal acidification, V-type ATPase may be involved in the removal of the organic enamel matrix to allow for a more complete, and greater volumetric, hydroxyapatite crystal formation [6].

The data presented in this current study represents in part an overall picture of the gene and protein expression portfolio of the V-type ATPase proton pump subunits in correlation to their possible function in matrix protein degradation during enamel formation. Our qPCR data comparing expression profiles during secretory-stage and maturation-stage amelogenesis was performed on the full range of V-type ATPase subunits, including all gene homologues for the various subunits. Interestingly, 11 out of 22 (50%) gene transcripts examined were significantly upregulated at maturation stage (Figure 1). The fact that multiple genes code for the same V-type ATPase subunit (e.g. Atp6v0a is produced from 3 genes, and Atp6v1g is also produced from 3 genes – see Table 1 for other examples) suggest that robust gene expression exists (multiple genes coding the same subunit and being expressed simultaneously); and that there are possible redundancies in same-functioning (multiple gene homologues for individual subunits) gene products. Suitable antibodies to study each of the V-type ATPase subunits in rodents are not widely available, therefore we selected four commercially-available antibodies for our studies; one for the V₀ subunit (Atp6v0d2), and three for the V₁ complex (Atp6v1b2, Atp6v1c1 and Atp6v1e1). Each of the four antibodies give similar (although not identical) spatiotemporal expression data, thus we believe that we can describe, with some accuracy, the spatiotemporal expression profile of the V-type ATPase pump throughout amelogenesis. As discussed by Josephsen et al. [6] the V-type ATPase activity is clearly upregulated from secretory-stage to maturation-stage amelogenesis, and appears most prominent in the ruffled border of ruffle-ended ameloblasts. Our current immunolocalization data supports these earlier findings of Josephsen et al. [6]. During enamel maturation V-type ATPase activities are not only limited to the ameloblasts, but also shows significant expression in the papillary layer cells. However, ameloblast do express higher levels of V-type ATPase subunits than do the cells of the papillary layer and may imply a greater involvement of maturation ameloblasts, rather than papillary layer cells, in endocytotic activities related to the removal of the extracellular enamel matrix [10].

Atp6v1c1 and Atp6v1e1 are two of the least studied V-type ATPase subunits, especially in conjunction to tooth development. In our study, both Atp6v1c1 and Atp6v1e1 subunits showed similar gene expression profiles. Both subunits were noticeably upregulated at maturation-stage enamel organ cells as observed at both the mRNA and protein levels. In the enamel organ, the immunoreaction for Atp6v1c1 and Atp6v1e1 subunits were located in ameloblasts, and to a lesser extent in papillary layer of maturation-stage enamel organs. The strongest expression of both the Atp6v1c1 and Atp6v1e1 subunits was detected in the apical ruffled border of RA, and in the cytoplasm of ameloblasts. As has been discussed, similar findings on V-type ATPase localization, using immunohistochemistry and immunoelectron microscopy, has been previously described [6,23]. Lin et al. proposed that the RA cells secreted protons to the enamel matrix, through the V-type ATPase proton pump, to activate hydrolytic enzymes involved in degradation of organic enamel matrix [23]. An alternative explanation was presented by Josephsen et al. who suggest a modulation in pH (i.e. as cells transition from ruffle ended ameloblasts to smooth ended ameloblasts) at the enamel surface compartment, may in fact keep the spaces between individual crystallites open to allow for the passage and ameloblast-mediated endocytosis of degraded enamel matrix proteins [6].

Additional studies are needed to decipher the apparent multiple roles of V-type ATPase in amelogenesis.

While our study was limited to the non-pathological expression profile of the V-type ATPase proton pump in the enamel organ cells, this proton pump has also been studied in disease states. Knockout animal models for some of these V-type subunits exhibit distinct disease-related phenotypes including osteoporosis, oculocutaneous albinism and impaired retinal pigmentation. Mutations in *Atp6v0a3* (also known as *Tcirg1*) have been proven to result in bone resorption defects, which, leads to osteopetrosis in both humans and mice [27,28,29,30,31]. In humans, mutations of the *Atp6v0a3* gene may also be responsible for human malignant infantile osteopetrosis [27,29,31]. Prior studies on *Atp6v0a3/Tcirg1* mutant mice have shown that all incisor and molar teeth developed normally; however, molar root development was severely disrupted and the incisors appear short when compared to the wild-type control mice [32]. *Atp6v0d2* has been identified as key player of osteoclast-specific proton pump mediating extracellular acidification in bone resorption. Cellular level analysis of *Atp6v0d2* expression showed that this subunit is not only expressed in the enamel organ but also odontoblast [10]. It will be interesting to examine *Atp6v0d2* knockout animal [33] to identify if there are any dentition malformations to help, in part, relate various ion channelopathies to dental health. *Atp6v1b* subunits (*Atp6v1b1* and *Atp6v1b2*) are probably the most extensively studied subunits of V-type ATPase. Diseases such as proximal and renal tubular acidosis associated with sensorineural deafness have been linked to mutations in *Atp6v1b1* [31,34]. In some situations the up-regulation of the *Atp6v1b2* subunit may compensate for the loss of the *Atp6v1b1* subunit, as has been observed in the renal medullary intercalated cells of *Atp6v1b1*-null animals [35].

In conclusion, the molecular basis for lysosomal and endosomal activity during amelogenesis is still only partially understood. The data presented here, in part, helps decipher the pH regulatory pathways associated with V-type ATPase during enamel formation. Further investigations to establish a link between systemic diseases involving the ion channels and ion transporters are still required to better understand how they can positively impact dental health.

MATERIAL AND METHODS

Rat tissue dissection

Four adult male Wistar Hannover rats weighing 170–190 g were euthanized and their mandibles were immediately dissected out and the surrounding excess soft tissues removed. Mandibles were then frozen in liquid nitrogen as previously described [36]. Mandibles were kept in liquid nitrogen overnight and the samples were then lyophilized for 24 h. The mandibular bone encasing the lower incisors was carefully removed to expose the entire labial surface of the enamel organ. Enamel organ cells were then collected by gentle scraping from the two different regions of the incisor as described in [5]. A definitive molar reference line isolated cells from secretory- and maturation-stages (here early-maturation and late-maturation enamel organ isolates were pooled and analyzed together) following the method previously described [5,37]. Tissues from both mandibular incisors (from one rat)

were pooled as a single sample, and a sample size of 4 rats (n=4) was used as biological replicates.

Total RNA isolation and real-time PCR

Total RNA from the enamel organ was extracted by homogenizing the freeze-dried cells of each secretory- and maturation-stage using Qiagen RNeasy Minikit. Reverse-transcribed PCR was performed using iScript cDNA Synthesis kit Thermal (Bio-Rad Life Sciences, Hercules, CA). Quantitative real-time PCR (qPCR) reactions were performed with iQ™ SYBR® Green Supermix (BioRad) by applying primer pairs shown in **Table 2**. All V-type ATPase subunits, and control transcript primer pairs were purchased from RealTimePrimers.com (<http://www.realtimeprimers.com>). Relative expression of mRNA was calculated using the delta delta CT method [38]. All values for the mRNA levels presented were normalized to β -actin. All qPCR samples from each of the 4 rats were run in triplicate and an average Cq value was considered for further calculations. Standard deviations are calculated for (n=4) and included in the graphs. Statistically significant changes ($p < 0.05$) in gene expression were determined using the two-tailed Student's t-test.

Western blot analysis

Enamel organ cells from secretory- and maturation-stages were collected from four 4-week old rats weighing ~ 100gm, pooled, lysed with RIPA buffer (1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, 50 mM Tris, pH 8.0) and protease inhibitor cocktail, complete mini (Roche Applied Sciences, Indianapolis, IN, USA). Samples were homogenized manually with a pestle, and then sonicated with a BRANSON digital sonifier Model 450 (All-Spec Industries, Wilmington, NC, USA) (10% intensity, 10 seconds on and 10 seconds off). Samples were then cleared by centrifugation (15,000 g, 15 min, 4°C). Proteins were quantified using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) and equal quantities were loaded (15 μ g per lane) onto 4–12% SDS–PAGE resolving gels. Antibodies applied were: mouse-derived anti-Gapdh (GA1R, Thermo Scientific, dilution 1:3,000); chicken-derived anti-Amelx [39] (dilution 1:1,000); rabbit-derived anti-Odam (dilution 1:500) [40]; anti-ATP6V0D2 (ab87059, Abcam, dilution 1:50), anti-ATP6V1B2 (ab73404, Abcam, dilution 1:100), anti-ATP6V1C1 (ab87163, Abcam, dilution 1:100) and anti-ATP6V1E1 (ab111733, Abcam, dilution 1:100). Secondary antibodies used were: horseradish peroxidase labeled goat-derived anti-mouse (Cat # 32430, Pierce Biotechnology); goat-derived anti-rabbit (Cat # 32460, Pierce Biotechnology); and rabbit-derived anti-chicken (Cat # 31401, Pierce Biotechnology). Labeled protein bands were detected with an Enhanced ChemiLuminescence (ECL) detection system (Pierce Biotechnology).

Immunohistochemistry

Mandibular incisor of 4-week old rats weighing ~100 g were dissected and fixed by perfusion (4% paraformaldehyde, 0.1% glutaraldehyde, 0.08mM sodium cacodylate, 0.05% calcium chloride, pH 7.2 – 7.4) overnight. The incisors were then decalcified in 4.13% disodium ethylenediaminetetraacetic acid (EDTA), at 4°C, for 2 months. The samples were embedded in paraffin and 4- μ m sections were cut with a microtome. The paraffin sections

were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched by 3% H₂O₂ in methanol for 10 min. Sections were blocked with 1% bovine serum albumin (BSA) and incubated overnight with a primary polyclonal antibody against ATP6V0D2 (ab87059, Abcam at a 1:500 dilution), ATP6V1B2 (ab73404, Abcam at a 1:1,000 dilution), ATP6V1C1 (ab87163, Abcam at a 1:2000 dilution) and ATP6V1E1 (ab111733, Abcam at a 1:2,000 dilution). After washing, sections were incubated with a biotinylated anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA). Sections were then incubated with Histostain-Plus kit (DAB) (Invitrogen) following manufacturer specifications. Sections were counter-stained with hematoxylin. Negative control sections were prepared in which no primary antibody was applied, but all other steps were performed.

Acknowledgments

The authors would like to thank Dr. Antonio Nanci (University of Montreal) for the antibody against Odam. The authors would also like to thank the two anonymous reviewers for their helpful comments. This work was supported by grant DE019629 from the National Institute of Dental and Craniofacial Research, National Institutes of Health.

Abbreviations

Actb	beta actin
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
Amelx	amelogenin
Enam	enamelin
Odam	odontogenic ameloblast associated
DMEM	Dulbecco's modified Eagle's medium
PN	post natal
RIPA buffer	radio-immunoprecipitation assay buffer
BCA	bicinchoninic acid
SDS	sodium dodecyl sulfate

REFERENCES

1. Sasaki S, Takagi T, Suzuki M. Cyclical changes in pH in bovine developing enamel as sequential bands. *Arch Oral Biol.* 1991; 36:227–231. [PubMed: 1877895]
2. Smith CE. Cellular and chemical events during enamel maturation. *Crit Rev Oral Biol Med.* 1998; 9:128–161. [PubMed: 9603233]
3. Duan X. Ion channels, channelopathies, and tooth formation. *J Dent Res.* 2014; 93:117–125. [PubMed: 24076519]
4. Lacruz RS, Nanci A, Kurtz I, Wright JT, Paine ML. Regulation of pH During Amelogenesis. *Calcif Tissue Int.* 2010; 86:91–103. [PubMed: 20016979]
5. Lacruz RS, Smith CE, Moffatt P, Chang EH, Bromage TG, Bringas P, et al. Requirements for ion and solute transport, and pH regulation during enamel maturation. *J Cell Physiol.* 2012; 227:1776–1785. [PubMed: 21732355]
6. Josephsen K, Takano Y, Frische S, Praetorius J, Nielsen S, Aoba T, et al. Ion transporters in secretory and cyclically modulating ameloblasts: a new hypothesis for cellular control of

preeruptive enamel maturation. *Am J Physiol Cell Physiol.* 2010; 299:C1299–1307. [PubMed: 20844245]

7. Bronckers AL, Lyaruu DM, Jansen ID, Medina JF, Kellokumpu S, Hoeben KA, et al. Localization and function of the anion exchanger Ae2 in developing teeth and orofacial bone in rodents. *J Exp Zool B Mol Dev Evol.* 2009; 312B:375–387. [PubMed: 19206174]
8. Lyaruu DM, Bronckers AL, Mulder L, Mardones P, Medina JF, Kellokumpu S, et al. The anion exchanger Ae2 is required for enamel maturation in mouse teeth. *Matrix Biol.* 2008; 27:119–127. [PubMed: 18042363]
9. Bronckers A, Kalogeraki L, Jorna HJ, Wilke M, Bervoets TJ, Lyaruu DM, et al. The cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in maturation stage ameloblasts, odontoblasts and bone cells. *Bone.* 2010; 46:1188–1196. [PubMed: 20004757]
10. Lacruz RS, Brookes SJ, Wen X, Jimenez JM, Vikman S, Hu P, et al. Adaptor protein complex 2-mediated, clathrin-dependent endocytosis, and related gene activities, are a prominent feature during maturation stage amelogenesis. *J Bone Miner Res.* 2013; 28:672–687. [PubMed: 23044750]
11. Katchburian E, Holt SJ. Role of lysosomes in amelogenesis. *Nature.* 1969; 223:1367–1368. [PubMed: 4185631]
12. Simmer JP, Fincham AG. Molecular mechanisms of dental enamel formation. *Crit Rev Oral Biol Med.* 1995; 6:84–108. [PubMed: 7548623]
13. Sun-Wada GH, Wada Y, Futai M. Lysosome and lysosome-related organelles responsible for specialized functions in higher organisms, with special emphasis on vacuolar-type proton ATPase. *Cell Struct Funct.* 2003; 28:455–463. [PubMed: 14745137]
14. Breton S, Brown D. Regulation of luminal acidification by the V-ATPase. *Physiology (Bethesda).* 2013; 28:318–329. [PubMed: 23997191]
15. Marshansky V, Rubinstein JL, Gruber G. Eukaryotic V-ATPase: novel structural findings and functional insights. *Biochim Biophys Acta.* 2014; 1837:857–879. [PubMed: 24508215]
16. Saroussi S, Nelson N. The little we know on the structure and machinery of V-ATPase. *J Exp Biol.* 2009; 212:1604–1610. [PubMed: 19448070]
17. Saroussi S, Nelson N. Vacuolar H(+)-ATPase-an enzyme for all seasons. *Pflugers Arch.* 2009; 457:581–587. [PubMed: 18320212]
18. Bonten EJ, Annunziata I, d'Azzo A. Lysosomal multienzyme complex: pros and cons of working together. *Cell Mol Life Sci.* 2014; 71:2017–2032. [PubMed: 24337808]
19. Mindell JA. Lysosomal acidification mechanisms. *Annu Rev Physiol.* 2012; 74:69–86. [PubMed: 22335796]
20. Miranda KC, Karet FE, Brown D. An extended nomenclature for mammalian V-ATPase subunit genes and splice variants. *PLoS One.* 2010; 5:e9531. [PubMed: 20224822]
21. Lacruz RS, Smith CE, Chen YB, Hubbard MJ, Hacia JG, Paine ML. Gene-expression analysis of early- and late-maturation-stage rat enamel organ. *Eur J Oral Sci.* 2011; 119(Suppl 1):149–157. [PubMed: 22243241]
22. Lacruz RS, Smith CE, Bringas P Jr. Chen YB, Smith SM, Snead ML, et al. Identification of novel candidate genes involved in mineralization of dental enamel by genome-wide transcript profiling. *J Cell Physiol.* 2012; 227:2264–2275. [PubMed: 21809343]
23. Lin HM, Nakamura H, Noda T, Ozawa H. Localization of H(+)-ATPase and carbonic anhydrase II in ameloblasts at maturation. *Calcif Tissue Int.* 1994; 55:38–45. [PubMed: 7922788]
24. Damkier HH, Josephsen K, Takano Y, Zahn D, Fejerskov O, Frische S, et al. Fluctuations in surface pH of maturing rat incisor enamel are a result of cycles of H(+)-secretion by ameloblasts and variations in enamel buffer characteristics. *Bone.* 2014; 60:227–234. [PubMed: 24373736]
25. Sasaki T, Tadokoro K, Yanagisawa T, Higashi S, Garant PR. H+-K+-ATPase activity in the rat incisor enamel organ during enamel formation. *Anat Rec.* 1988; 221:823–833. [PubMed: 2847591]
26. Lacruz RS, Smith CE, Kurtz I, Hubbard MJ, Paine ML. New paradigms on the transport functions of maturation-stage ameloblasts. *J Dent Res.* 2013; 92:122–129. [PubMed: 23242231]

27. Frattini A, Orchard PJ, Sobacchi C, Giliani S, Abinun M, Mattsson JP, et al. Defects in TCIRG1 subunit of the vacuolar proton pump are responsible for a subset of human autosomal recessive osteopetrosis. *Nat Genet.* 2000; 25:343–346. [PubMed: 10888887]
28. Li YP, Chen W, Liang Y, Li E, Stashenko P. Atp6i-deficient mice exhibit severe osteopetrosis due to loss of osteoclast-mediated extracellular acidification. *Nat Genet.* 1999; 23:447–451. [PubMed: 10581033]
29. Michigami T, Kageyama T, Satomura K, Shima M, Yamaoka K, Nakayama M, et al. Novel mutations in the $\alpha 3$ subunit of vacuolar H(+)-adenosine triphosphatase in a Japanese patient with infantile malignant osteopetrosis. *Bone.* 2002; 30:436–439. [PubMed: 11856654]
30. Scimeca JC, Franchi A, Trojani C, Parrinello H, Grosgeorge J, Robert C, et al. The gene encoding the mouse homologue of the human osteoclast-specific 116-kDa V-ATPase subunit bears a deletion in osteosclerotic (oc/oc) mutants. *Bone.* 2000; 26:207–213. [PubMed: 10709991]
31. Sobacchi C, Frattini A, Orchard P, Porras O, Tezcan I, Andolina M, et al. The mutational spectrum of human malignant autosomal recessive osteopetrosis. *Hum Mol Genet.* 2001; 10:1767–1773. [PubMed: 11532986]
32. Bronckers AL, Lyaruu DM, Bervoets TJ, Medina JF, DenBesten P, Richter J, et al. Murine ameloblasts are immunonegative for Tcirl1, the v-H-ATPase subunit essential for the osteoclast plasma proton pump. *Bone.* 2012; 50:901–908. [PubMed: 22245629]
33. Lee SH, Rho J, Jeong D, Sul JY, Kim T, Kim N, et al. v-ATPase V0 subunit d2-deficient mice exhibit impaired osteoclast fusion and increased bone formation. *Nat Med.* 2006; 12:1403–1409. [PubMed: 17128270]
34. Borthwick KJ, Kandemir N, Topaloglu R, Kornak U, Bakkaloglu A, Yordam N, et al. A phenocopy of CAII deficiency: a novel genetic explanation for inherited infantile osteopetrosis with distal renal tubular acidosis. *J Med Genet.* 2003; 40:115–121. [PubMed: 12566520]
35. Paunescu TG, Russo LM, Da Silva N, Kovacicova J, Mohebbi N, Van Hoek AN, et al. Compensatory membrane expression of the V-ATPase B2 subunit isoform in renal medullary intercalated cells of B1-deficient mice. *Am J Physiol Renal Physiol.* 2007; 293:F1915–1926. [PubMed: 17898041]
36. Smith CE, Nanci A, Moffatt P. Evidence by signal peptide trap technology for the expression of carbonic anhydrase 6 in rat incisor enamel organs. *Eur J Oral Sci.* 2006; 114(Suppl 1):147–153. [PubMed: 16674677]
37. Smith CE, Nanci A. Secretory activity as a function of the development and maturation of ameloblasts. *Connect Tissue Res.* 1989; 22:147–156. 1989. [PubMed: 2598666]
38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
39. Shapiro JL, Wang H, Wen X, Tannukit S, Paine ML. An amelogenin minigene to study alternative splicing. *DNA Cell Biol.* 2006; 25:1–5. [PubMed: 16405395]
40. Moffatt P, Smith CE, St-Arnaud R, Nanci A. Characterization of Apin, a secreted protein highly expressed in tooth-associated epithelia. *J Cell Biochem.* 2008; 103:941–956. [PubMed: 17647262]

Highlights

The V-type ATPase proton pump is highly expressed during enamel maturation.

23 genes code for V-type ATPase, and 11 of these are upregulated during enamel maturation.

V-type ATPase is more highly expressed in ameloblasts than in the papillary layer.

V-type ATPase is located both in the cytoplasm and the apical membrane of maturation ameloblasts.

Figure 1. Real-time RT-PCR results for gene expression profiles of V-type ATPase subunits at successive stages of enamel organ development

Results of real-time PCR analyses for all V-type ATPase associated genes. From rat enamel organ epithelial cells the mRNA expression profile of each gene is analyzed at secretory and maturation stages, and normalized to β -actin. Enam and Odam are used here as an additional control to examine the quality of isolated RNA. For primers used see Table 2. The averages and standard error of deviation were based on three independent real-time PCR amplifications using RNA from three different preparations. The Student's *t*-test compared the expression of each gene between secretory and maturation stages. An asterisk (*) shows significance at $p < 0.05$. When a significant change is observed, the fold change (maturation-stage relative to secretory-stage) is noted and labeled above the asterisk.

Figure 2. Protein expression of selected V-type ATPase subunits in enamel organ of rat incisors
 Western blot analyses are shown using protein extracts from secretory-stage and maturation-stage of rat enamel organ epithelial cells. Western blot analysis of the expression of Amelx and Odam, used here as additional controls, is noted in rat enamel organ. Gapdh used here as a loading or normalizing control. Antibodies recognizing Atp6v0d2, Atp6v1b2, Atp6v1c1 and Atp6v1e1 gave a band corresponding to a molecular weight predicted protein product. Antibodies were deemed suitable for additional immunolocalization studies.

Figure 3. Immunolocalization of Atp6v0d2 subunit in enamel organ of rat incisors

Sagittal sections of mandibular incisors from 4-wk-old rats were stained with antibodies against subunit Atp6v0d2 and then counterstained with haematoxylin (Panel A). Differential localization is shown at secretory (panel a1), and maturation (panel b1) stages of enamel organ development. Panels a2 and b2 are higher magnification images of enamel organ regions in panels a1 and b1 respectively. Enamel organ cells; ameloblasts (Am), stratum intermedium (Si, and black arrowhead), stellate reticulum (Sr), and papillary layer cells (PL)

are identified. Dentin (Den), enamel or enamel space (En) and connective tissue (CT) also labeled. Scale bars are included.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4. Immunolocalization of Atp6v1b2 subunit in enamel organ of rat incisors

Sagittal sections of mandibular incisors from 4-wk-old rats were stained with antibodies against subunit Atp6v1b2 and then counterstained with haematoxylin (Panel A). Differential localization is shown at secretory (panel a1), and maturation (panel b1) stages of enamel organ development. Panels a2 and b2 are higher magnification images of enamel organ regions in panels a1 and b1 respectively. Enamel organ cells; ameloblasts (Am), stratum intermedium (Si, and black arrowhead), stellate reticulum (Sr), and papillary layer cells (PL)

are identified. Dentin (Den), enamel or enamel space (En) and connective tissue (CT) also labeled. Scale bars are included.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5. Immunolocalization of Atp6v1c1 subunit in enamel organ of rat incisors

Sagittal sections of mandibular incisors from 4-wk-old rats were stained with antibodies against subunit Atp6v1c1 and then counterstained with haematoxylin (Panel A). Differential localization is shown at secretory (panel a1), and maturation (panel b1) stages of enamel organ development. Panels a2 and b2 are higher magnification images of enamel organ regions in panels a1 and b1 respectively. Enamel organ cells; ameloblasts (Am), stratum intermedium (Si, and black arrowhead), stellate reticulum (Sr), and papillary layer cells (PL)

are identified. Dentin (Den), enamel or enamel space (En) and connective tissue (CT) also labeled. Scale bars are included.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. Immunolocalization of Atp6v1e1 subunit in enamel organ of rat incisors

Sagittal sections of mandibular incisors from 4-wk-old rats were stained with antibodies against subunit Atp6v1e1 and then counterstained with haematoxylin (Panel A). Differential localization is shown at secretory (panel a1), and maturation (panel b1) stages of enamel organ development. Panels a2 and b2 are higher magnification images of enamel organ regions in panels a1 and b1 respectively. Enamel organ cells; ameloblasts (Am), stratum intermedium (Si, and black arrowhead), stellate reticulum (Sr), and papillary layer cells (PL)

are identified. Dentin (Den), enamel or enamel space (En) and connective tissue (CT) also labeled. Scale bars are included.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Rodent genes coding for the V-type ATPase subunits.

V₀ domain subunits	Genes
Atp6v0a	<i>Atp6v0a1</i> <i>Atp6v0a2</i> <i>Atp6v0a3/Tcirg1</i>
Atp6v0b	<i>Atp6v0b</i>
Atp6v0c	<i>Atp6v0c</i>
Atp6v0d	<i>Atp6v0d1</i> <i>Atp6v0d2</i>
Atp6v0e	<i>Atp6v0e1</i> <i>Atp6v0e2</i>

V₁ domain subunits	Genes
Atp6v1a	<i>Atp6v1a</i>
Atp6v1b	<i>Atp6v1b1</i> <i>Atp6v1b2</i>
Atp6v1c	<i>Atp6v1c1</i> <i>Atp6v1c2</i>
Atp6v1d	<i>Atp6v1d</i>
Atp6v1e	<i>Atp6v1e1</i> <i>Atp6v1e2</i>
Atp6v1f	<i>Atp6v1f</i>
Atp6v1g	<i>Atp6v1g1</i> <i>Atp6v1g2</i> <i>Atp6v1g3</i>
Atp6v1h	<i>Atp6v1h</i>

Table 2

Primer list used for real-time PCR of the V-type ATPase subunits.

Gene ID	Forward primer 5'-3'	Reverse primer 3'-5'	Product (bp)
<i>Actin</i>	ACAGGATGCAGAAGGAGATTAC	TCTATCCTGGCCTCACTGT	117
<i>Enam</i>	CCAGCAAGCATCCTACCTAATC	GAGATGATGTGCCTACTGTGAG	101
<i>Odam</i>	CAGGCTAGTCCTATGTCCTATGT	TTTACATGCTTCTGCCCTGG	96
<i>Atp6v0a1</i>	CTCCTTCCTGTGACTCTTGTTTC	CTAACCTGTCCGTTCTCACTTC	86
<i>Atp6v0a2</i>	CTGAGACGGGTGAAGTCATAAA	CTGTTACCACTGCCACATCT	108
<i>Atp6v0a3</i>	CTACAAGCTGAGTCCCTTCAC	CTGAGGCAGGAGAGGAATAAAG	103
<i>Atp6v0b</i>	GGAAGAGAAGGTAGGTGCATTAT	AAACTACCACGCAGGCTAC	116
<i>Atp6v0c</i>	CTAGAGTGCGCCTTGTGTATAAG	CTATTCTGTGTCTGTGGAGCA	103
<i>Atp6v0d1</i>	GCACCTGCAGAGTACAGATTAT	GAAGATGGTAGTAGAGTTCCGC	109
<i>Atp6v0d2</i>	TCATCTCCAGACCACTGACTAT	GGAGATGAGGAAGAAGCTATGC	97
<i>Atp6v0e1</i>	AGGTGCTGTTCTGGGAAATC	AACATGAGAGTCAGCGATGTG	94
<i>Atp6v0e2</i>	CTGTCTGCTGTACCTCTTCTG	GAATGAGACCATCTGGTACGTG	95
<i>Atp6v1a</i>	TCCGTAGCCTGGAAGATTAGA	CTCATCTCCAACCCTTTGCT	97
<i>Atp6v1b1</i>	ACAGCCAGGTTCTTCAAGTC	AATGACCCTACGATTGAGCG	95
<i>Atp6v1b2</i>	GGCGAGTGGAAGGTAGAAATG	CTATCCCTGACTTGACTGGGTA	97
<i>Atp6v1c1</i>	CGTCCGTGACTTCCAGTATAAC	AAGAAGCAGTTTGGACCACT	96
<i>Atp6v1c2</i>	CCTGGAGAACTTGGAGAAGAAA	AATATCTCATAACCCTGCTGGTC	115
<i>Atp6v1d</i>	GGTCGAAACCTCCTGAAGAAA	GAATAAGATGCTGATGGGTGAG	96
<i>Atp6v1e1</i>	GATGGCTTTCATCGAACAAGAAG	GAAAGGTCGTCTTGTGCAAAC	99
<i>Atp6v1e2</i>	GAGGAAGGTGTACGTGTTCTATG	AGAACCCAGAAGTCGTCAAAG	117
<i>Atp6v1f</i>	CCACCCTAATTTCCTGGTAGTG	CTCATCAACCAGTACATCGCA	118
<i>Atp6v1g1</i>	GAAGCAGCCAGCCATATAGT	CCTAACCCAAGTCACTAGATGC	109
<i>Atp6v1g2</i>	CTCGGTGTCCTCATCTTTGTAG	CATAGTTCAGATGGGTAGTGG	111
<i>Atp6v1g3</i>	CCACAGAAAGAGTCGGGATAG	CATTTCTCCTGGATGGCACT	138
<i>Atp6v1h</i>	GTGAACACCTTCGTCGTATA	TCATTCTGCAGCATTCCGTA	102