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IDENTIFICATION OF NOVEL CANDIDATE GENES INVOLVED IN MINERALIZATION OF DENTAL ENAMEL BY GENOME-WIDE TRANSCRIPT PROFILING

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Abstract

The gene repertoire regulating vertebrate biomineralization is poorly understood. Dental enamel, the most highly mineralized tissue in mammals, differs from other calcifying systems in that the formative cells (ameloblasts) lack remodeling activity and largely degrade and resorb the initial extracellular matrix. Enamel mineralization requires that ameloblasts undergo a profound functional switch from matrix-secreting to maturational (calcium transport, protein resorption) roles as mineralization progresses. During the maturation stage, extracellular pH decreases markedly, placing high demands on ameloblasts to regulate acidic environments present around the growing hydroxyapatite crystals. To identify the genetic events driving enamel mineralization, we conducted genome-wide transcript profiling of the developing enamel organ from rat incisors and highlight over 300 genes differentially expressed during maturation. Using multiple bioinformatics analyses, we identified groups of maturation-associated genes whose functions are linked to key mineralization processes including pH regulation, calcium handling and matrix turnover. Subsequent qPCR and Western blot analyses revealed that a number of solute carrier (SLC) gene family members were up-regulated during maturation, including the novel protein Slc24a4 involved in calcium handling as well as other proteins of similar function (Stim1). By providing the first global overview of the cellular machinery required for enamel maturation, this study provide a strong foundation for improving basic understanding of biomineralization and its practical applications in healthcare.

Keywords

enamel maturation; genome-wide analysis; mineralization

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INTRODUCTION

A defining characteristic of vertebrate development is the transformation of soft gel-like extracellular matrices into hard, mineralized structures of the skeleton. A common physiological pathway to produce these structures is through the ability of cells to channel calcium and phosphate ions to specific sites where mineral is deposited allowing crystal growth (Smith, 1998). An important challenge for mammals is dealing with the acidic conditions at crystal growth sites resulting from proton build up during the deposition of ions when creating hydroxyapatite (Hap). This is particularly evident in the development of dental enamel, the most mineralized tissue in mammals. Despite the fundamental importance of these critical tasks, both the identity of the required cellular machinery and the molecular mechanisms by which ameloblast cells orchestrate these processes remain unclear.

Enamel development involves two main functional stages, secretion and maturation (Nanci, 2008). In the secretory stage, ameloblasts are highly polarized cells that synthesize and secrete a number of structural enamel matrix proteins (EMP) in a temporally constrained manner. The bulk of the enamel tissue develops during the secretory stage in a protein-rich environment maintained at near-neutral pH conditions (Lacruz et al., 2010a; Smith, 1998; Smith et al., 2005; Smith et al., 1996). In the extracellular space, thin Hap crystals elongate under the influence of EMPs and are also influenced by the movements of the ameloblasts away from the dentine. In contrast, maturation stage cells are specialized for transport, show less protein synthesis, and have a greater capacity for endocytotic activity (Nanci, 2008; Smith, 1998). EMP expression markedly decreases at this stage as crystals greatly expand in width and thickness generating acidic conditions induced by massive proton release (Simmer and Fincham, 1995). Ameloblasts are thus required to orchestrate the transport of solutes and ions to the maturing enamel to allow crystal growth and control excess acidification (Lacruz et al., 2010a; Simmer and Fincham, 1995; Smith, 1998).

As they transition to the maturation stage, ameloblast perform the three broad functions of regulating calcium flux, maintaining acid-base balance and removing the EMPs. To survey the genetic repertoire used to drive these key elements of enamel mineralization, we undertook genome-wide transcript profiling of rat incisor enamel organ, comparing the secretory and maturation stages. Our results identified a broad set of candidate genes associated with enamel maturation, providing novel insights into the machinery used by enamel cells to transport calcium.

MATERIALS AND METHODS

Rat Tissue Dissections

We obtained samples from two different Wistar Hannover rat populations (Group 1 = 10 animals (180g) and Group 2 = 5 animals (100g)) using previously described methods (Lacruz et al., 2011a). Immediately after the animals were euthanized, their mandibles were dissected out and surrounding soft tissues removed. The mandibles were placed in liquid nitrogen, on average, within 5 minutes of the initial dissection. After storing the samples overnight in liquid nitrogen, we lyophilized them for 24 hours and then carefully removed the mandibular bone encasing the lower incisors to expose the entire labial surface. A molar reference line was used to isolate enamel organ cells from secretory, early-mid and from mid-late maturation as described (Smith and Nanci, 1989). We here focus on the comparison between secretory and mid-late maturation stages. Comparisons between early-mid and mid-late maturation from these sampled rat populations were reported elsewhere (Lacruz et al., 2011a). Cells dissected from the rat enamel organ include ameloblasts, but may also contain other cell types such as papillary layer cells, small blood vessels and some connective tissue. All vertebrate animal manipulation complied with Institutional and Federal guidelines.

To collect protein samples for analysis, four rats weighing ~100g were euthanized and their mandibles dissected out, which were then placed in ice. The bone surrounding the labial surface of the incisors was removed to isolate enamel organ cells which were collected by gentle scraping. Cells were obtained from secretory and maturation stages using the molar reference line as above (Smith and Nanci, 1989). A rat pancreas, heart and lung tissue were also isolated.

Genome-wide Expression Profiling

RatRef-12-v1 Expression BeadChips (Illumina, Inc., San Diego, CA, USA) were used to interrogate approximately 22,000 transcripts selected primarily from the National Center for Biotechnology Information (NCBI) RefSeq database (Release 16). Gene expression profiling analyses were conducted on Group 1 and Group 2 samples independently, each in quadruplicate and on separate BeadChips. All sample preparation, hybridization, BeadChip processing, and data acquisition were performed at the Southern California Genotyping Consortium (SCGC) according to the manufacturers recommended protocols. The raw data from each animal group were processed independently in the Bioconductor R environment using a newly optimized method (Shi et al., 2010) to produce normalized log(2) transformed gene expression scores for each developmental stage, as detailed in Tables S1 and Table S2. A gene was taken to be differentially expressed if both Groups showed a consistent: (i) >1.2-fold change in the geometric means of the gene expression scores and (ii) two-tailed Student's t-test $P < 0.05$ when subjected to Benjamini and Hochberg (B-H) correction for multiple comparisons (Benjamini and Hochberg, 1995) among the technical replicates in the group.

Bioinformatics analysis

WebGestalt software was used to conduct GeneOntology, KEGG, transcription factor binding site, and miRNA enrichment analyses (Kirov et al., 2007; Zhang et al., 2005). Ingenuity Pathways Analysis (IPA) v8 software (Ingenuity Systems, Redwood City, CA, USA) was used to analyze functional relationships of differentially expressed genes (DEGs).

Quantitative PCR

Total RNA was extracted from enamel organ by homogenizing the freeze-dried cells using the Qiagen RNeasy Minikit and generated corresponding cDNAs using the iScript cDNA Synthesis kit (Bio-Rad Life Sciences, Hercules, CA). Real-time, quantitative-PCR (qPCR) was performed with iQTM SYBR[®] Green Supermix (Bio-Rad Life Sciences) using rat-specific primer pairs. All primer pairs were designed to span intronic regions using rat orthologs of either the human or mouse qPCR primer pair sequences identified using "PrimerBank" (<http://pga.mgh.harvard.edu/primerbank/index.html>) or designed by SABioSciences (<http://www.sabiosciences.com/>). Relative mRNA expression levels were calculated using the delta-delta C_T method (Livak and Schmittgen, 2001) with all values normalized relative to β -actin.

Antibodies

A rabbit anti-mouse Amelx, rabbit anti-human LAMP1, and rabbit anti-human STIM2 polyclonal antibodies were obtained from Abcam (catalog numbers ab54507, ab24170 and 59344 respectively). A rabbit anti-human STIM1 and rabbit anti-human ACTB monoclonal antibodies was obtained from Epitomics (catalog numbers 3250-1 and 1854-1 respectively) whereas a rabbit anti-human SLC24A4 polyclonal antibodies from ProteinTech Group (catalog number 18992-1) and chicken anti-human GAPDH polyclonal antibodies were obtained from Chemicon (catalog number AB2302). We used the manufacturers recommended concentration for all Western blot analyses.

Western blotting

Cell lysates were prepared in ice-cold RIPA (1% NP40, 0.1% SDS, 0.5% deoxycholate, 150mM NaCl, 50mM Tris pH 8.0) protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail tablets from Roche). We homogenized the samples using a pestle prior to sonication, which we then cleared by centrifuging at 16,000 rpm for 15 minutes at 4°C. Proteins were quantitated using Micro BCA Protein Assays (Pierce) and equally loaded at 15µg/lane and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels. Results were quantitated by densitometry using ImageJ.

RESULTS

Identification of Differentially Expressed Genes (DEGs)

To compare transcript profiles in secretory and mid-late maturation stages of enamel development, we have analyzed rat incisor enamel organ. Using strict selection criteria (B-H corrected $P < 0.05$, ≤ 1.2 -fold), a total of 686 and 614 transcripts were found to be differentially expressed (up-or down-regulated) in either of the two specimen groups (i.e. 180g and 100g animals). Of these, a total of 166 transcripts were identified as being consistently up-regulated during maturation and 155 identified as being down-regulated, in both groups (Appendix 1 and Appendix 2).

GeneOntology (GO) Analysis

To analyze broad patterns in the data, we conducted Gene Ontology (GO) analyses of the DEGs shared between secretory and mid-late maturation stages in the two animal groups. We used stringent criteria (Fisher's exact test B-H corrected $P < 0.05$, minimum of five genes) for selection of enriched categories. The enriched categories of up-regulated DEGs in mid-late maturation included response to metal ions (11 genes, B-H corrected $P = 0.0002$), response to inorganic substance (12 genes, B-H corrected $P = 0.0002$), ion binding (41 genes, B-H corrected $P = 0.0018$), ion transmembrane transporter activity (15 genes, B-H corrected $P = 0.00019$), active transmembrane transporter activity (11 genes, B-H corrected $P = 0.0018$), symporter activity (6 genes, B-H corrected $P = 0.0019$), response to stress (29 genes, B-H corrected $P = 0.0053$). GO analysis also highlighted specific cellular components including extracellular region part, plasma membrane part and vesicles in this DEG group (Table S3). Relevant enriched categories of down-regulated DEGs in maturation relative to the secretory stage included odontogenesis (5 genes, B-H corrected $P = 0.0019$), negative regulation of cell proliferation (11 genes, B-H corrected $P = 0.0004$), pattern specification process (7 genes, B-H corrected $P = 0.03$), inflammatory response (7 genes, B-H corrected $P = 0.034$), regulation of signal transduction (13 genes, B-H corrected $P = 0.039$), and regulation of cell proliferation (12 genes, B-H corrected $P = 0.039$) (see Table S4).

KEGG Analysis

We also conducted KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analyses of the DEGs shared in both animal groups. As above, we used stringent criteria (Fisher's exact test B-H corrected $P < 0.05$) for selection of enriched categories. Enriched categories of DEGs up-regulated in mid-late maturation compared to the secretory stage included lysosome-associated pathway (*Cd68*, *Lamp1*, *Slc11a2*, *Ctsk*, *Tcirl1*, and *Laptm5*), metabolic pathways (*Amacr*, *Ugcg*, *Qprt*, *Xdh*, *Pfkf*, *Arg1*, *Anpep*, *Upp1*, *Enpp1*, *RGD1562690*, *Atp6v1b2*, *Tm7sf2*, *Tcirl1*, *Tpi1*, *Pla2g7*, *Bcl2a1d*, and *Ndufb2*), leukocyte transendothelial migration (*Cldn4*, *Ptk2b*, *Cyba*, *Mmp9*, and *Cldn10*) and cell adhesion molecules (*Cldn4*, *RT1-Db1*, *Sdc1*, *RT1-Da*, and *Cldn10*) (see Table S5). The two KEGG pathways enriched for the down-regulated DEGs were basal cell carcinoma and hedgehog signaling pathway (*Bmp2*, *Shh*, and *Hhip* in both instances) (Table S6).

Gene Network Analysis

We used Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, California) to analyze functional relationships among DEGs. The top five networks identified were: i) Cell Death, Connective Tissue Development and Function, Dermatological Diseases and Conditions, ii) Cellular Movement, Dermatological Diseases and Conditions, Genetic Disorder, iii) Small Molecule Biochemistry, Connective Tissue Disorders, Inflammatory Disease iv) Carbohydrate Metabolism, Immunological Disease, Lipid Metabolism, and v) Molecular Transport, Nucleic Acid Metabolism, Small Molecule Biochemistry. A graphical representation of the top network is shown in Fig. S1. The most down-regulated networks were: i) Connective Tissue Development and Function, Skeletal and Muscular System Development and Function, Tissue Development, ii) Cellular Movement, Tumor Morphology, Cancer, iii) Carbohydrate Metabolism, Drug Metabolism, Small Molecule Biochemistry, iv) Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry, and v) Molecular Transport, Small Molecule Biochemistry, Cell Cycle. A graphical representation of the top down-regulated network is shown in Fig. S2.

Transcriptional Factor (TF) Binding Site Analysis

In order to explore the higher-order transcriptional regulation of DEGs, we screened for the possible enrichment of transcription factor binding sites in their promoters. The up-regulated DEGs in mid-late maturation were enriched for Ap1 (26 genes, B-H corrected $P=3.9 \times 10^{-8}$), Maz (30 genes, B-H corrected $P=0.0002$), and Nfat binding sites (5 genes, B-H corrected $P=0.03$) among others (Table S7). The over-representation of Nfat binding sites (associated with the *Usp2*, *Klf5*, *Ndr2*, *Snap25*, and *Ctsk* genes) is in keeping with the role of this calcium regulator and the high activity of Ca^{2+} associated genes reported here (Appendix 1). The down-regulated DEGs in mid-late maturation were enriched for Foxo4 (24 genes, B-H corrected $P=0.0016$), Areb6 (8 genes, B-H corrected $P=0.002$), Bach2 (7 genes, B-H corrected $P=0.004$), and Lef1 binding sites (22 genes, B-H corrected $P=0.002$) among others (Table S8).

MicroRNA Target Enrichment Analysis

MicroRNAs or miRNAs consists of ~22 nucleotide non-coding RNAs that negatively regulate their target genes through direct degradation of the messenger RNA (mRNA) and/or translational inhibition (Jovanovic et al., 2010). Over 1400 miRNA are known in the human genome (Kozomara and Griffiths-Jones, 2011). To further explore the regulation of DEGs, we screened for miRNAs known to regulate their expression. Three miRNA targets were identified as being important in maturation stage relative to secretory stage (Table S9). These are rno_AATGTGA (MIR-23A, MIR-23B) which included (*Atp6v1b2*, *Ptk2b*, *Slc1a1*, *Sec14l1*, *Klf5*, *Lamp1*, *Slc6a14*, *Slc6a14*, *Slc6a14*, and *Pkp4*), rno_CTGAGCC (MIR-24) included (*Sema4a*, *Arhgef5*, *Sesn1*, *Gpx3*, and *Rab5c*) and rno_TTTGCAC (MIR-19A, MIR-19B) which included (*Atp6v1b2*, *Ptk2b*, *Slc6a8*, *Slc24a4*, *Sdc1*, *Ptp4a1*, *Tnfrsf12a*, and *Tp53inp1*). Two miRNAs were identified as down-regulated in maturation including rno_CTACTGT, MIR-199A (*Bmp2*, *Cd24*, *Plod2*, *Fnbp1l*, and *Plekhh1*) and rno_CTTTGTA, MIR-524 (*Prickle1*, *Slc17a6*, *Col14a1*, *Ndel1*, *Olfm1*, *P4ha2*, *Fnbp1l*, and *Atp2b1*) (Table S10).

qPCR analysis reveals selective up-regulation of Slc family during maturation and Stim1

Noting that *Stim1* and 8 different members of the solute carrier (SLC) gene family were up-regulated during maturation (Appendix 1), we undertook a more detailed analysis of *Stim1* and several transcripts using qPCR (Figure 1). Relative levels of *Stim1* increased 8-fold in maturation. Focusing on Slc genes, it was found that the most highly expressed genes were *Slc4a4*, *Slc24a4* and *Slc34a2* with fold changes of 3.3-fold, 23-fold and 64-fold respectively.

Other SLC genes with lesser expression were *Slc1a1* and *Slc39a2* showing fold changes of 24-fold and 21-fold respectively. None of the SLC genes sampled here by qPCR were down-regulated. To validate our qPCR data, we analyzed expression levels of enamel proteins known to undergo differential expression during maturation. As expected, we found that expression levels of *Amelx*, *Enam* and *Mmp20* decreased by 70-fold, 57-fold and 28-fold respectively, whereas the enamel protease (*Klk4*) was up-regulated by 15-fold, in keeping with their known expression profiles (Bartlett et al., 2006; Hu et al., 2002; Lu et al., 2008; Nanci et al., 1998; Simmer and Fincham, 1995; Simmer et al., 2009; Simmer et al., 2010).

Western blotting shows maturation-associated up-regulation of two novel calcium-handling proteins

Recognizing that one of the up-regulated SLCs (*Slc24a4*) encodes for NCKX4, a distinctive type of plasma membrane acting as a reversible $\text{Na}^+/\text{Ca}^{2+}$ exchanger dependent on K^+ not previously reported in enamel cells, we extended the analysis to protein level using Western blot analysis (Figure 2). Antibodies to *Slc24a4* revealed a band of the expected mass and which was about 2-fold stronger during maturation when compared to secretory stage (Figure 2). Comparison of maturation stage enamel cells with other tissues revealed similar abundance to pancreas, whereas levels of *Slc24a4* were greater in lung compared to heart as noted elsewhere by Northern blot (Li and Lytton, 2002). A parallel analysis was applied to the candidate calcium-entry proteins, the stromal interacting molecules *Stim1* and *Stim2*. We measured the expression levels of *Stim1* by densitometry and found that it showed nearly 9-fold increase relative to the secretory stage, in keeping with the up-regulation observed in microarray analysis. *Stim2* protein expression was up-regulated in maturation by about 1.5-fold as measured by densitometry. We concluded that *Slc24a2*, *Stim1* and *Stim2* are all strong candidate proteins for performing a key calcium-handling role during enamel mineralization. Finally, following our BeadChip results we analyzed the expression of the lysosomal membrane protein *Lamp1*, which was up-regulated 1.2-fold in maturation.

DISCUSSION

Enamel biomineralization has been viewed commonly with a focus on the development of the bulk enamel, which takes places in the secretory stage. Less attention has been paid overall to the processes that regulate the final stages of mineral growth and protein degradation and resorption, which occur during the maturation stage (Smith, 1998). To investigate the cellular machinery responsible for regulating the final stages of enamel mineralization, we screened the rat incisor enamel organ to identify candidate genes involved in this process. The fact that over 300 genes were identified as being up- or down-regulated from secretory to mid-late maturation indicated that this functional switch involves a profound change at the transcriptome level. The affected genes can be assigned to the areas of matrix turnover, calcium handling, pH and ion regulation. The relevance of genes identified by this study is discussed with a focus on these three functional areas and their relevance in dental disease.

Network and Pathway Analysis reveals maturation-associated functions

Our analyses of potential networks involved in regulating the transition from secretory to maturation indicated that cell death, cellular movement and molecular transport are highly represented networks in maturation. It has been known for quite some time that about 25% of the ameloblasts that functioned during the secretory stage die as part of transition into the maturation stage, and another 25% die progressive over time throughout the maturation stage (Smith and Warshawsky, 1977). Many of the genes represented in our analysis are involved in cell death. Of particular interest is the elevated expression of *Mt3*, which in brain

tissues has been associated with neuronal cell death through release of toxic levels of Mt3-bound intracellular metals, such as zinc, in response to oxidative stress (Lee et al., 2003). At present, it is unknown which signals or processes dictate the massive apoptosis of ameloblasts as they enter maturation. The high expression of *Mt3*, perhaps associated with other stress inducers such as *Sesn1* or *Sod1* (Appendix 1), may be relevant in this regard. Indeed response to stress was one of the most relevant biological processes detected in our GO analysis (Table S3). Other important cellular functions and biological processes in GO analysis showed that general transport-associated functions were enriched as the enamel organ engages in the final stages of mineralization. Key potential transporters and their functions are discussed below. Importantly, KEGG pathway analysis (Table S5) highlighted that lysosomal degradation and cell adhesion were relevant pathways in maturation. We discuss these pathways in the context of matrix turnover.

1) Matrix turnover—The onset of enamel maturation involves a marked decrease in expression of the major EMPs, namely amelogenin (*Amelx*) (which forms > 90% of secreted EMPs), ameloblastin (*Ambn*) and enamelin (*Enam*) (Bartlett et al., 2006; Smith, 1998). *Mmp20* and *Klk4* are two proteases responsible for EMP degradation are expressed primarily during either secretion or maturation, respectively (Bartlett and Simmer, 1999; Ryu et al., 1999). As a first step, we analyzed the expression of known secretory stage genes. As expected, our DEG lists showed strong down-regulation for *Amelx*, *Enam*, *Mmp20* and up-regulation of *Klk4* during maturation in both Groups 1 and 2. *Ambn* was also >2.4-fold down-regulated in Groups 1 and 2, but only met our B-H corrected $P < 0.05$ criterion in the later group. We concluded that our microdissected enamel organ specimens provided an appropriate source for further investigation of maturation-associated changes in gene expression. This was also confirmed by qPCR analysis of four selected EMP transcripts (i.e. *Amelx*, *Enam*, *Mmp20*, and *Klk4*).

Enamel crystal growth in maturation requires that proteinase-based breakdown and resorption/removal of organic material for the final expansion of width and thickness of enamel crystallites (Smith et al., 2011). However, the cause versus effect relationships of these events remains unclear: does crystal growth stimulate removal of residual proteins or is protein removal a requirement for crystal growth (Nanci and Smith, 1992; Smith et al., 2011). With regards to matrix break down, a novel serine protease known as caldecrin (*Ctrc*) was identified as markedly up-regulated during maturation in groups 1 and 2 (17.1 and 4.4-fold respectively). Genetic lesions involving existing EMP proteases and EMPs account only for about a third of amelogenesis imperfecta (AI) cases, leaving many new AI genes to be discovered (Wright, 2006). The candidate role(s) of *Ctrc* in enamel maturation and AI are now under further investigation in our laboratories (Lacruz et al., 2011c).

Ameloblasts clearly participate in EMP removal, but how organic material passes from the ECM to the endosomal/lysosomal system is poorly understood (Shapiro et al., 2007). Several endocytosis-related proteins (*Cd68*, *Laptn5*, *Lamp1*, and *Tcirg1*) were up-regulated during maturation in both groups. Besides their primary association with lysosomal and endosomal membranes, some of these proteins are also located on the plasma membrane. They are involved with trafficking between the plasma membrane and endosomal/lysosomal structures through associations with one or more adaptor protein complexes. The expression of the lysosomal proton pump (*Atp6v1b2*) was up-regulated in both groups, consistent with a potential role in EMP removal. The observed up-regulation of proteolytic and endocytotic machinery during the mid-late maturation stage is in keeping with current understanding of the requirements for proper enamel maturation by ameloblasts in which essentially all of the EMP scaffold is removed to allow complete mineral growth (Smith, 1998). These data clearly warrant additional analysis of these genes in maturation stage and further clarification of how endocytosis is regulated in the enamel organ.

2) Calcium handling—The domination amongst up-regulated genes of transcripts putatively associated with Ca^{2+} transport and handling is clearly indicative of the importance of Ca^{2+} in the final stages of enamel maturation (Hubbard, 2000). Enamel organ cells must handle large quantities of calcium ions (Ca^{2+}) both for extracellular mineralization and intracellular signaling purposes (Hubbard, 2000). Ca^{2+} -handling proteins comprised a significant proportion of DEGs that were up-regulated during mid-late maturation, consistent with previous biochemical studies indicating a high Ca^{2+} -binding capacity of enamel organ cells during maturation (Hubbard, 1995; Hubbard, 1996). Based on their gene expression profiles, we identified a variety of proteins not previously associated with tooth development that may play key roles during enamel formation (Appendix 1). Several genes of the calcium binding *S100* gene family were identified, of which the most dominant was *S100a4* (Appendix 1). One or more of these *S100* genes might correspond to the unidentified 12kDa Ca^{2+} -binding protein associated with enamel maturation (Hubbard, 1996). The *S100g* gene was also markedly up-regulated in the maturation stage, but it did not meet our criteria for DEG. The up-regulation of *S100g* (calbindin-9kDa) also accords with previous studies (Franklin et al., 2001; Hubbard, 2000). Perhaps an even more interesting finding is the up-regulation of *Rcan* and *Stim1* during maturation. Given the high expression levels of calcineurin previously reported (Hubbard, 1995), the marked up-regulation of *Rcan1* (*aka* Dscr1) during maturation is of particular interest with regard to how enamel organ cells avoid Ca^{2+} toxicity (Franklin et al., 2001; Hubbard, 1998; Hubbard, 2000). *Rcan1*/calcineurin signaling has emerged as a key protective mechanism against Ca^{2+} -mediated stress (Ermak and Davies, 2002) and so it might be pivotal during maturation when Ca^{2+} -handling demands are most critical. Interestingly, the *Nfat* family of transcription factors (the main downstream effectors of calcineurin) is thought to play a fundamental role in bone and cartilage development (Wu et al., 2007).

Considering the entry and extrusion steps of Ca^{2+} transport, both of which are poorly understood (Hubbard, 2000), it is interesting that *Slc24a4*, a distinct type of $\text{Na}^+/\text{Ca}^{2+}$ exchanger that co-transport K^+ , expression markedly up-regulated in mid-late maturation at the transcript (Appendix 1) and protein levels (Figure 2). This might explain previous difficulties associating conventional $\text{Na}^+/\text{Ca}^{2+}$ exchangers (NCX family) with enamel maturation (Lacruz et al., 2011b; Okumura et al., 2010; Tye et al., 2010). Our recent analysis on *Ncx1* and *Ncx3* gene expression in rat incisor enamel organ indicates that their expression either does not change from secretory to maturation (*Ncx1*), or that it decreases (*Ncx3*) (Lacruz et al., 2011b). The increases levels of *Stim1* protein, which recently was implicated in replenishing ER/ Ca^{2+} stores (Deng et al., 2009), introduces a potential mechanism for Ca^{2+} entry and further substantiates the ER as having a key role in Ca^{2+} transport across enamel organ cells (Hubbard, 2000; Turnbull et al., 2004). The expression of *Stim2* was also up-regulated during maturation and although it did not meet our criteria for DEG, given its similar function to that of *Stim1*, we analyzed protein expression for *Stim2*. Both *Stim1* and *Stim2* protein expression levels increased in maturation relative to secretory stage (Figure 2). One other aspect concerning Ca^{2+} requiring further discussion is the regulation of Ca^{2+} activity (Hubbard, 2000; Smith, 1998). The most highly up-regulated gene in maturation (~200-fold) was the neuropeptide cholecystokinin (*Cck*), which has been linked to Ca^{2+} signaling (Cancela, 2001) and therefore it becomes a good candidate gene for studying this function in the enamel organ. Cadherin17 (*Cdh17*), a Ca^{2+} -dependent cell adhesion molecule, was also up-regulated. Interestingly, an intracellular receptor for inositol (*Itp1*) involved in Ca^{2+} release from ER, is markedly down-regulated in mid-late maturation (>10-fold in both experiments).

3) pH control and ion transport—We have recently addressed elsewhere the challenges and requirements for regulating extracellular pH during amelogenesis (Lacruz et al., 2010a;

Lacruz et al., 2010b; Lacruz et al., 2011b), an area of study that has been also revisited by others (Bronckers et al., 2010; Josephsen et al., 2010; Urzua et al., 2011; Zheng et al., 2011). Enamel organ cells must regulate many other ions in the ECM besides Ca^{2+} , including protons, phosphate and metals. The fact that abnormal expression of various genes involved in pH control results in severe enamel defects (Chang et al., 2011; Lacruz et al., 2010b; Lyaruu et al., 2008; Wright et al., 1996) highlights the relevance of maintaining pH homeostasis for mineral crystallite expansion during mineral maturation. One of the most widely represented gene families in our analysis is the solute carrier (SLC) protein superfamily, which comprises approximately 380 genes arranged over 50 families (HUGO Gene Nomenclature Committee). SLCs serve both actively and passively as the main gate keepers in mammalian cells controlling uptake and efflux of ions (Hediger et al., 2004). Our data suggests that is also likely the case for cells of the enamel organ. We found 8 SLC proteins to be up-regulated during enamel maturation (*Slc1a1*, *Slc6a8*, *Slc11a2*, *Slc16a3*, *Slc24a4*, *Slc26a1*, *Slc34a2* and *Slc39a2*) (Appendix 1), including transporters for H^+ , Na^+ , Cl^- , K^+ , PO_4^{3-} , Zn^{2+} and Ca^{2+} . Most of these have not been associated previously with enamel formation. Real time-PCR confirmed our BeadChip results (Figure 1). The *Slc6* gene family in general regulates levels of extracellular solute concentrations, but more specifically *Slc6a8* is involved in creatine transport, critically important in maintaining ATP homeostasis (Chen et al., 2004). *Slc34* genes are involved sodium/phosphate transport, with *Slc34a2* being commonly localized to the apical pole of epithelial cells (Murer et al., 2004). The high up-regulation of *Slc34a2* (Appendix 1), is of major interest. It is known that phosphate rapidly enters the enamel layer at a high rate relative to ruffled-ended ameloblasts (Kawamoto and Shimizu, 1990; McKee et al., 1989). The potential discovery of a likely phosphate transporter functioning in the enamel organ is relevant to understanding the regulation of such channels in enamel mineralization. *Slc39* genes are involved with transport of metal ions, being *Slc39a2* involved in zinc transport (Eide, 2004). At least two anion exchangers were identified including *Slc4a2* and the *Slc26a1*. This latter gene has been localized to the basolateral pole of kidney cells acting as a sodium independent SO_4^{-2} -anion transporter possibly involved in HCO_3^- transport (Mount and Romero, 2004), but has not been implicated in enamel formation. Reports also suggest that increase of SO_4^{-2} transport is activated by extracellular Cl^- (Mount and Romero, 2004). Insofar as chloride transport is concerned, the cystic fibrosis conductance transmembrane regulator (*Cftr*) and *Clc* genes were the only known chloride channels in enamel cells, and at least in the case of *Cftr*, it appears indispensable for proper enamel maturation (Chang et al., 2011; Hou et al., 2008; Lacruz et al., 2010a; Sui et al., 2003). The identification of *Clca2* (a Ca^{2+} regulated Cl^- channel) and *Clc2* (an intracellular Cl^- channel) and their marked up-regulation during maturation (Appendix 1) suggests that Cl^- transport in enamel requires alternative mechanisms to enable Cl^- in and out of cells. However, the role of *Clca2* as a potential regulator of cell adhesion cannot be ruled out (Loewen and Forsyth, 2005).

The anion exchanger *Slc4a2* is often functionally coupled with the *Cftr* Cl^- channel, which also is also up-regulated in maturation stage ameloblasts (Chang et al., 2011). These proteins might work in concert with carbonic anhydrases, two of which (*Car6* and *Car2*) are expressed to a high degree in maturation (Lacruz et al., 2011b). Carbonic anhydrases, which play a key role in pH regulation by mediating production of bicarbonate, have previously been implicated in enamel mineralization (Lacruz et al., 2010a; Supuran, 2008). Interestingly, *Slc11a2* has been linked to transferrin-dependent iron transport from endosomes to the cytosol (Kozomara and Griffiths-Jones, 2011). Furthermore, ferritin (*Ftl*), a major storage for soluble non-toxic iron, was also up-regulated in late maturation consistent with its involvement in pigmentation of the enamel in rodent incisors (McKee et al., 1987; Smith, 1998). Finally, in the context of transcellular transport being dependent on tight intercellular junctions, it is noteworthy that claudin-10 (*Cldn10*) and claudin-4 (*Cldn4*),

involved in the development of tight junctions (Inai et al., 2008), were more highly expressed during mid-late maturation (Appendix 1).

Down-regulated SLCs during mid-late maturation included *Slc17a6* and *Slc17a9* (sodium-dependent inorganic phosphate cotransporters), *Slc7a5* (cationic amino acid transporter), *Slco1a5* (amino acid transporter), *Slco4a1* (anion exchanger). Other ion channels down-regulated include the *Fxyd4* which modulates Na²⁺-K-ATPases, and *Clic6*, an intracellular chloride channel (Appendix 2).

4) Enamel health and disease—Several of the enamel matrix proteins identified here showing significant change between secretory and maturation stage, when mutated, result in enamel pathologies both in animal models and in the human population. The prime examples are *AMELX*, *ENAM*, *AMBN*, *MMP20* and *KLK4* (Wright, 2006; Wright et al., 2009). Less information is available for how other genes identified in this study may impact enamel development and pathology. However, abnormal enamel phenotypes have been reported as a result of *STIM* and *Orai* mutations (Feske, 2009; Feske, 2010; McCarl et al., 2009; Picard et al., 2009), *Slc4a2/AE2* (Gawenis et al., 2004; Lyaruu et al., 2008) and carbonic anhydrase 2 (*CA2*) (Awad et al., 2002; Nagai et al., 1997; Strisciuglio et al., 1990). In addition, enamel pathology has been linked recently to *WDR72* (El-Sayed et al., 2009) and *FAM83H* mutations (Kim et al., 2008). While the expression of neither of these causal gene candidates was significantly changed, the related *Fam3c* gene was down-regulated while the *Fam129b* (aka LOC36211 aka *Minerva*) and *Fam82a1* (aka LOC313840) genes were up-regulated in mid-late maturation. One of the known functions of *Fam129b* *in vitro* is the inhibition of apoptosis (Chen et al., 2011), which contrasts with the top network function identified in maturation which was cell death (Figure S1). Clearly a greater understanding of the molecular function of these additional enamel-related genes is needed to further our understanding of enamel mineralization. Our genome-wide analysis of the enamel-mineralizing transcriptome draws attention to many proteins that may have a critical role to play in enamel formation. Additional studies are now needed to substantiate such mechanistic relationships.

MicroRNAs have been analyzed in conditional knock outs of *Dicer1*, a deletion that results in severe enamel defects (Cao et al., 2010). This study highlighted the benefit of studying the role/s of miRNAs in tooth development, a field of study that is only just emerging (Cao et al., 2010). Tables S9 and S10 provide a full list of candidate miRNAs associated with the enamel organ of healthy rats at the secretory and mid-late maturation stages of enamel formation. Based on our observed gene expression profiles, these may be especially promising candidates involved in regulating various stages of amelogenesis.

Gene regulation in various stages of enamel maturation

In a previous study comparing cells collected from the early-mid and mid-late enamel maturation phases obtained from the same Group 1 and Group 2, a list of 66 differentially expressed genes was identified (Lacruz et al., 2011a). To further provide a comprehensive analysis linking these data with our current analysis and to better define changes in gene regulation between secretory and the two maturation stages, Figure S3 provides a Venn diagram summarizing DEGs in comparisons of all three phases of enamel formation. Table S11 provides the identities of all DEGs.

CONCLUSION

In this study, we exploited the two-step process of enamel formation to achieve a comprehensive molecular survey of the enamel organ to identify the genetic events driving enamel mineralization. Astonishingly, over 160 genes increased expression from the

secretory to mid-late maturation stages. We have assigned these genes to functional units and interpreted their putative roles in maturation based on bioinformatic analyses. Many of these genes might be involved in the intraand extracellular regulation of enamel mineralization. This highlights the power of genomic approaches for the discovery of genes and gene networks in biomineralization. It also suggests that additional combined genetic and epigenetic characterizations (Wang et al., 2010) and evolutionary analyses (MacQuarrie et al., 2011; Wold and Myers, 2008) could provide a useful means to identify and prioritize genomic elements relevant to enamel biology. Our findings open the door to a vast array of mechanistic exploration and research avenues that may lead to a better understanding of biomineralization and its practical applications in healthcare.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. qPCR analyses of Slc genes selected based on BeadChip data
Relative expression levels of 14 Slc genes were assessed by qPCR comparing cells dissected from secretory and maturation stages. As observed, the most highly expressed gene is *Slc24a4*, which also shows a marked fold change increase. Other relevant genes include *Slc4a4* and *Slc34a2*, with fold changes of 3.3-fold and 64-fold respectively. All data was normalized to β -actin. One way ANOVA was used to assess significance between secretory and maturation stage for each transcript. Significance ($p < 0.05$) is marked by an asterisk. Fold changes for each gene are included.

Figure 2. Western blot analysis of protein expression levels in secretion and maturation stages
 For selected gene transcripts identified in the microarray, we performed Western blot analysis to correlate protein levels to mRNA levels at the two stages of amelogenesis. Freshly dissected cells from the secretory and maturation stages of rat incisors were used as a source to analyze protein expression. **A)** The pattern of amelogenin (Amelx) expression, being absent in maturation stage enamel organ cells but significantly expressed in secretory stage, and also absent in pancreas, heart and lung, is in keeping with its normal expression pattern. Microarray and qPCR results (Table 1) indicate that Lamp1, Stim1 and Slc24a4 experienced an increase in expression at the maturation stage. **B)** Densitometric analysis of protein expression relative to Actb. A marked increase in protein level of Stim1 (~8.6 fold)

and to a lesser extent Slc24a4 (~2 fold). Although Stim2 was not included in the final list of up-regulated genes based on stringent data filtering (Appendix 1), raw intensity signals had indicated a noticeable fold increase. Stim2 protein expression levels increased by ~1.5-fold whereas Lamp1 increased 1.2-fold. Pancreas, heart and lung tissues, and Actb and Gapdh antibodies were used as controls. The antibody used for Actb from Epitomics does not recognize heart Actb.