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The role of epithelial *Stat3* in amelogenesis during mouse incisor renewal

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Abstract

The aim of this study was to evaluate the role of epithelial Signal transducer and activator of transcription 3 (STAT3) in mouse incisor amelogenesis. Since *Stat3* is expressed in the epithelial component of developing and adult mouse teeth, we generated and analyzed *Krt14*$^{Cre/+}$;*Stat3*$^{fl/fl}$ mutant mice in which *Stat3* was inactivated in epithelia including ameloblast progenitors and ameloblasts – the cells responsible for enamel formation. Histological analysis showed little enamel matrix in mutant incisors compared to controls. Delayed incisor enamel mineralization was demonstrated using micro-computed X-ray tomography (microCT) analysis and was supported by an increase in the pre-expression distance of enamel-enriched proteins such as amelogenin (AMEL), ameloblastin (AMBN), and Kallikrein4 (KLK4). Lastly, scanning electron microscopy (SEM) analysis showed little enamel mineralization in mutant incisors underneath the mesial root of the 1st molar, however, the micro-architecture of enamel mineralization was similar in the erupted portion of control and mutant incisors. Taken together, our findings demonstrate for the first time that the absence of epithelial *Stat3* in mice leads to delayed incisor amelogenesis.

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CONFLICT OF INTEREST

All authors declare that there are no conflicts of interest.

Keywords

dental enamel; amelogenesis; incisor; mice; transcription factor

INTRODUCTION

Enamel is the hard, outer covering of teeth and is the only physiologic mineralized tissue, which includes bone, dentin, and cementum to be generated from ectoderm-derived epithelial cells. The mouse incisor provides a valuable model to study the molecular and cellular mechanisms of enamel formation or amelogenesis, because unlike the rooted mouse molar, the incisor renews continuously throughout life fueled by mesenchymal and epithelial stem cells [Jheon et al., 2013]. Enamel is deposited in an asymmetric fashion exclusively on the labial (toward the lip) surface allowing abrasion of the enamel-free, lingual (toward the tongue) surface thereby maintaining incisors a consistent length and sharpness.

Dental epithelial stem cells (DESCs) fuel mouse incisor renewal and are located in niches called the labial and lingual cervical loops (laCL and liCL, respectively) located at the proximal end of the incisor (Fig. 1A,A') [Smith and Warshawsky, 1975a, b; Harada et al., 1999; Seidel et al., 2010; Juuri et al., 2012; Li et al., 2012; Biehs et al., 2013; Jheon et al., 2013]. The laCL comprises several different cell types including the stellate reticulum (SR), the outer enamel epithelium (OEE), the inner enamel epithelium (IEE), and the transit-amplifying (TA) region, the last of which eventually differentiate into enamel-generating ameloblasts (Fig.1A'') [Warshawsky and Smith, 1974]. The regulation of these various cell populations in the laCL involves major signaling pathways, including Bmp, Eda, Fgf, Notch, Shh, and Wnt family members, which also regulate mouse molar development [Bei and Maas, 1998; Mitsiadis et al., 1998; Harada et al., 1999; Harada et al., 2002; Millar et al., 2003; Wang et al., 2004; Wang et al., 2007; Felszeghy et al., 2010; Liu et al., 2010; Jheon et al., 2013]. Relatively little is known about the liCL.

Signal transducers and activators of transcription (STATs) comprise a unique family of transcription factors that transmit the interactions of cytokines, hormones, and growth factors with their cell surface receptors into transcriptional programs. STAT3 is widely expressed in different types of tissues and cells [Stepkowski et al., 2008], and plays key roles in early development evidenced by embryonic lethality when *Stat3* is globally inactivated in mice [Takeda et al., 1997]. STAT3 also plays crucial roles in a variety of biological functions including cell growth, migration, suppression and induction of apoptosis, and cell motility [Akira, 2000; Levy and Lee, 2002], regulates inflammation and immunity [Raz et al., 1999], and plays an important role in bone marrow cell differentiation [Chakraborty et al., 1999]. In addition, STAT3 has been implicated in oncogenesis as activated STAT3 has been associated with many human malignancies and the overexpression of a constitutively active form of STAT3 resulted in the transformation of fibroblasts [Bromberg et al., 1999; Couronne et al., 2013; Bruserud et al., 2015].

Despite a large body of work involving STAT3 in normal and cancerous cells [Abroun et al., 2015], relatively little is known about its role in tooth development. We embarked on this study because of the potential interaction of STAT3 with Islet1 (ISL1), a protein that was

recently shown to play a role in enamel mineralization during mouse incisor development and renewal [Naveau et al., 2017]. We determined that *Stat3* is expressed in the epithelial component of the developing tooth and continuously renewing incisor. Therefore, we generated mice in which *Stat3* was inactivated in epithelial tissues [Dassule et al., 2000; Zappia et al., 2016]. *Krt14*^{Cre};*Stat3*$^{fl/fl}$ mutant mice appeared normal and healthy, with the exception of a delay in incisor enamel mineralization. Thus, we conclude that epithelial *Stat3* is critical for the proper timing of incisor amelogenesis.

MATERIAL AND METHODS

Animals

Mice carrying the *Krt14*^{Cre} [Tg (*KRT14*-cre)1Amc] allele [Dassule et al., 2000] were mated with mice carrying the *Stat3* flox (*Stat3*^{tm2Aki}) allele [Takeda et al., 1998] to generate epithelial-specific, *Stat3*-inactivated (K*rt14*$^{Cre/+}$;*Stat3*$^{fl/fl}$) mutant mice. *Stat3*$^{fl/fl}$ mice were used as controls. Mice were maintained in a temperature-controlled facility with access to food and water *ad libitum*. Up to five mice of the same sex were housed together until time of sacrifice and no adverse events were reported. Both male and female mice were analyzed. All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at University of California, San Francisco, CA, USA and the mice were handled in accordance with the principles and procedure of the Guide for the Care and Use of Laboratory Animals under the approved protocol AN084146.

Histology and immunofluorescence labeling

Mice at embryonic day (E) 14.5, E16.5, postnatal day (P) 1, and 6 weeks were euthanized following standard IACUC protocol, collected, fixed in 4% paraformaldehyde at 4°C for 48h, demineralized in 0.5M EDTA for 2–14 days as needed, dehydrated, embedded in paraffin wax, and serially sectioned at 7μm. Histological sections were stained with haematoxylin and eosin (H&E). Immunofluorescence labeling was performed according to standard protocols. Antigen retrieval was performed by boiling the slides in Trilogy (Cell Marque) for 15 min and cooled at room temperature for 20 min after de-paraffinization and rehydration. Primary antibodies used were as follows: anti-rabbit STAT3 (1:200; Cat#7197, ProSci, used to detect STAT3 expression in wildtype specimens), anti-rabbit STAT3 (Phospho-Stat3 (Tyr705) (D3A7), Cat#9145S, Cell signaling, used to detect STAT3 expression in *Stat3*$^{fl/fl}$ and K*rt14*$^{Cre/+}$;*Stat3*$^{fl/fl}$ mice), anti-rabbit AMELX (amelogenin; 1:200; Cat#ab54507, Abcam), anti-rabbit AMBN (ameloblastin; 1:200; Cat#ab116347, Abcam), anti-kallikrein-4 or KLK4 (1:200; Abcam, Cat#ab71234, Abcam), enamelin or ENAM (1:200; Cat#sc-33107, Santa Cruz), and KI67 (1:200, Cat#RM-9106, Thermo Scientific). Goat anti-rabbit AlexaFluor 488 (1:500; Cat#A11008, Invitrogen) or 555 secondary antibody was used (1:500, Cat#A21428, Invitrogen).

Microscopy

Fluorescent and bright field images were taken using a DM5000B microscope with a DFC500 camera (Leica). For confocal images, an SP5 Upright Confocal microscope (Leica) was used.

Micro-computed X-ray tomography (MicroCT)

MicroCT was performed on a MicroXCT-200 (Xradia, Pleasanton, CA, USA) through the MicroCT Imaging Facility at UCSF. Each specimen was scanned at 75 KVp and 6W at 4× magnification. The opacity or density of enamel was measured using ImageJ 1.44o software (NIH) using the Plot Profile function.

Scanning Electron Microscopy (SEM)

Mouse hemi-mandibles were dissected free of soft and connective tissue, fixed in 4% PFA in PBS overnight, then dehydrated in a graded ethanol series and dried in a vacuum desiccator. Hemi-mandibles were then embedded in epoxy resin (resin 105 and hardener 205 at a ratio of 5:1 w/w, WestSystem, Bay City, MI, USA), ground to the desired thickness on a plate grinder (EXAKT 400CS, Norderstedt, Germany) using 800 grit silicon carbide paper and polished with 2000 and 4000 grit silicon carbide paper (Hermes Abrasives, Mississauga, ON, Canada). The exposed tissue was etched with 10% phosphoric acid for 30 seconds, rinsed with water and dried in a vacuum desiccator. Samples were mounted on SEM stubs with carbon tape, surfaces coated with 7nm gold using a sputter coating machine (Desk II, Denton Vacuum, Moorestown, NJ, USA), and imaged in a Philips SEM instrument (XL30 ESEM, Philips, Andover, MA, USA) operating at a beam energy of 20 keV in secondary electron or backscatter mode. Images were processed using Adobe Photoshop CS6 to adjust upper and lower limits of input levels in grayscale mode, and to apply auto balance and auto contrast settings.

Statistical analysis

All experiments were performed independently at least three times (i.e., N=3), and when applicable, presented as an average ± standard error of the mean. Student t-test was used to determine p-values and $p<0.01$ (**) and $p<0.05$ (*) were deemed to be significant.

RESULTS

Expression of *Stat3 in* wild-type mouse incisors and molars

We analyzed the expression profile of STAT3 in wild-type mouse heads (Fig. 1B–I). STAT3 was present in the dental epithelia of developing incisors and molars at E14.5, E16.5, P1, and in the incisor of 6-week old mice, as well as in the alveolar bone surrounding the developing teeth (Fig.1B–I). As expected STAT3 was not present in 6-week molars since ameloblasts are sloughed off once molars are erupted, unlike mouse incisors (Fig. 1I).

Stat3 mutant incisors show little or no enamel matrix

Since global inactivation of *Stat3* causes early embryonic lethality [Takeda et al., 1997], we generated conditional mice carrying an epithelial-specific deletion of *Stat3*. $Krt14^{Cre}$;$Stat3^{fl/fl}$ mutant mice were viable and appeared healthy overall similar to $Stat3^{fl/fl}$ control mice (data not shown). Since only the C-terminus of STAT3 is absent in mutant mice rendering the protein inactive, the absence of epithelial STAT3 was confirmed using a C-terminus-specific STAT3 antibody (Fig. S1A,B). Notably some mesenchymal STAT3 remained in mutant mice as expected. H&E staining of control and mutant adult, mandibular

incisors showed little or no enamel matrix in mutants compared to control (Fig. 1J–K'). We noted similar phenotypes in maxillary incisors (data not shown). No differences were detected in the liCL (Fig. 1J,K).

Incisor enamel mineralization is delayed in *Stat3* mutant mice

To analyze enamel mineralization, we performed microCT analysis on hemi-mandibles in 6-week old mice (Fig. 2). We did not detect any gross morphological differences in the incisor (Fig. 2A,H) or the molars (Fig. 2B,I). However, we observed that incisor enamel mineralization was delayed. *In silico* coronal, sequential sections of control (Fig. 2C–F) and mutant (Fig. 2J–M) hemi-mandibles at the level of the 1st (Fig. 2C,C',J,J'), 2nd (Fig. 2D,K) and 3rd (Fig. 2E,L) molars showed that mineralized enamel was readily detected and differences observed at the level of the 1st molar mesial cusp in controls (Fig. 2C') compared to mutants (Fig. 2J'). Erupted incisor enamel at the gingival junction (Fig. 2F,M) showed no difference in enamel between control and mutant incisors. These observations were quantified and confirmed by measuring incisor enamel opacities at the level of the 1st molar and erupted enamel (Fig. S1C–G). Furthermore, the distance between mineralized incisor enamel and the distal aspect of the 3rd molars in high-contrast microCT images revealed significant differences and confirmed the delay in mutant enamel mineralization (Fig. 2G,N). Interestingly, mineralized dentin also appeared to be affected in mutants, observed to be thinner in mutants compared to controls (Fig. 2C',J' and Fig. S1C,D).

The delay in incisor enamel mineralization was supported at the molecular level by immunofluorescence staining (Fig. 3 and Fig. S2). First, the proliferating cells in the TA region were detected using anti-KI67 antibody (Fig. 3A,B) and we determined that the length of the proliferative (i.e., TA) zone (Fig. 3C) and percentage of proliferating cells (Fig. 3D) were both slightly but significantly increased in mutants relative to controls. Second, the expression of amelogenin (AMEL), ameloblastin (AMBN), and kallikrein4 (KLK4), three enamel-enriched proteins essential for enamel matrix formation and mineralization, was significantly delayed in mutant incisors as evidenced by the increases in pre-expression distances (i.e., distance from the laCL to the start of AMEL/AMBN/KLK4 expression) (Fig. 3E–I and Fig. S2). Notably, a fourth enamel-enriched protein, enamelin (ENAM) also showed a slight delay in expression although it was not deemed to be significant ($p=0.07$; Fig. S2C–E).

SEM analysis confirms a delay in enamel mineralization in *Stat3* mutants

To determine whether inactivation of *Stat3* led to changes in enamel mineral architecture, we performed SEM analysis on control and mutant incisors in 3 distinct areas: 1) incisor enamel at the level of the mesial cusp of the 1st molar; 2) incisor enamel at the gingival margin; 3) molar enamel between the 1st and 2nd molars (Fig. 4). Mutant incisor enamel underneath the 1st mandibular molar showed little evidence of mineralization compared to controls, where the enamel rods extended from the dentino-enamel junction (DEJ) to the surface with inter-rod prisms located between the enamel rods (Fig. 4A,D). The microarchitecture of the incisor enamel at the gingival aspect looked similar in control and mutant mice suggesting that once mutant incisor enamel is mineralized it is similar to control incisor enamel (Fig.

4B,E). We did not detect any significant differences in molar enamel micro-architecture between control and mutant mice (Fig. 4C,F).

DISCUSSION

Little is known about *Stat3* and its role in teeth, specifically in tooth development. Previous studies showed that mutations in Stat3 cause hyper IgE syndrome (HIES) or Job's syndrome, a rare form of immunodeficiency [Grimbacher et al., 1999; Holland et al., 2007; Minegishi, 2009]. Most patients with this disease possess associated craniofacial defects including the retention of deciduous teeth, non- or ectopic eruption of permanent teeth, and delayed tooth eruption [Esposito et al., 2012]. *Stat3* has also been shown to be important in bone development and metabolism [Li, 2013], and thus may be a critical factor in orthodontic tooth movement [Zhou et al., 2016]. STAT3 also plays essential roles in the development of embryonic stem cells [Raz et al., 1999] and in the differentiation of dental pulp stem cells [Feng et al., 2016].

Krt14^{Cre};*Stat3*$^{fl/fl}$ mutant mice showed defective incisor enamel matrix production visualized by histology (Fig. 1J–K') and a delay in incisor enamel mineralization as shown by microCT and SEM analyses (Fig. 2,4 and Fig. S1). The delay in enamel mineralization was supported by increases in the TA region and proliferating cells (Fig. 3A–D), as well as increases in the pre-expression distances of ameloblast-enriched factors, AMEL, AMBN, KLK4, and ENAM (Fig. 3E-1 and Fig. S2), all suggestive of a delay in ameloblast differentiation. SEM analysis of mutant incisors demonstrated enamel hypo-mineralization underneath the 1st molar, but at the gingival margin where the incisor erupts into the oral cavity, enamel mineralization appeared to be normal with no differences detected in enamel micro-architecture between control and mutant incisors (Fig. 4).

Since epithelial inactivation of *Stat3* led to hypo-mineralized enamel on the labial incisor surface largely due to delayed differentiation of ameloblasts, but did not affect the enamel of rooted molars, *Stat3* may be an important regulator of DESCs. There is a large body of work that shows alterations in *Stat3* leads to changes in cell proliferation and differentiation in normal and cancerous cells [Abroun et al., 2015]. The specific effect of epithelial *Stat3* inactivation on mouse incisors but not molars also supports a potential role for *Stat3* in the development of anterior teeth (e.g., incisors and canines) but not posterior teeth (e.g., premolars and molars). However, it is also likely, but yet unclear, whether molar enamel development was also delayed. In addition, epithelial-specific *Stat3*-inactivation led to a change in incisor dentin (Fig. 2A1',B1') further demonstrating the importance of epithelial-mesenchymal interactions during enamel and dentin formation [Balic and Thesleff, 2015]. Further studies are required to determine the signaling pathways that are affected by the loss of epithelial *Stat3* during enamel and dentin formation.

In conclusion, our findings demonstrate that *Stat3* plays an important role in amelogenesis. Specifically, *Stat3* is expressed in developing mouse incisors and molars, and its expression is maintained in adult incisors. Epithelial-specific inactivation of *Stat3* led to a delay in ameloblast differentiation as demonstrated by the delayed expression of AMEL and AMBN,

and ultimately delayed incisor enamel mineralization. Thus, *Stat3* is important in the proper timing of mouse incisor amelogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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LIST OF ABBREVIATIONS

AMBN	ameloblastin
AMEL	amelogenin
DESCs	dental epithelial stem cells
ENAM	enamelin
IEE	inner enamel epithelium
KLK4	kallikrein4
laCL	labial cervical loop
liCL	lingual cervical loop
OEE	outer enamel epithelium
SEM	scanning electron microscopy
SI	stratum intermedium
SR	stellate reticulum
TA	transit-amplifying

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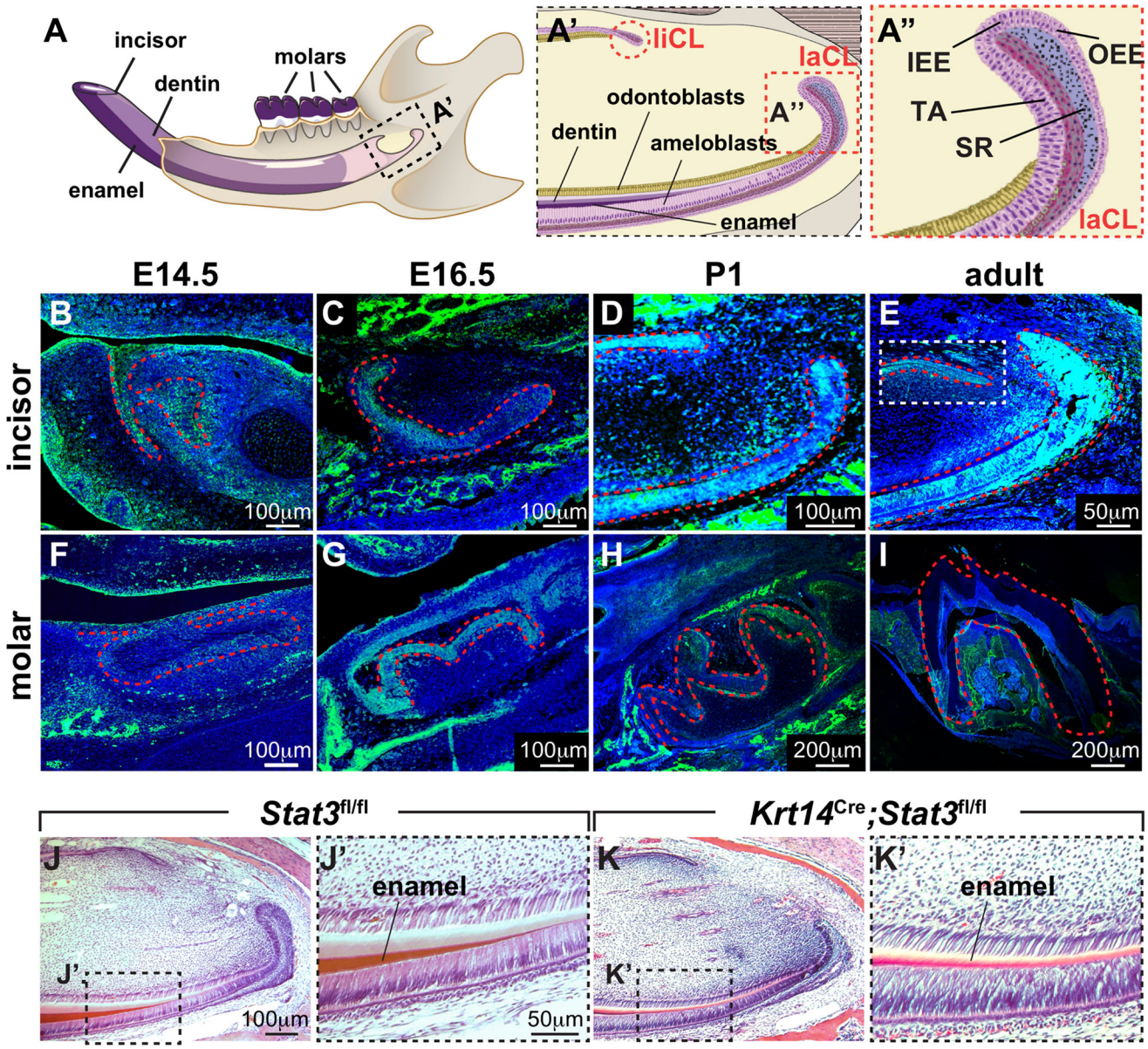

Fig. 1. *Stat3* is expressed in mouse mandibular teeth and enamel matrix altered in mutant incisors

(A) Illustration of the adult mouse hemi-mandible showing the incisor and molars, as well as the mineralized dentin and enamel in the incisor. (A') The proximal region of the incisor denoting the labial and lingual cervical loop (laCL and liCL, respectively, highlighted by dashed, red lines). (A'') Magnified illustration of the laCL indicating the outer enamel epithelium (OEE), inner enamel epithelium (IEE), transit amplifying (TA) region, and stellate reticulum (SR). (B–I) Immunofluorescence staining for STAT3 in wildtype mouse teeth (sagittal views) at E14.5, E16.5, P1, and 6 weeks showed the presence of STAT3 in developing incisors and molars, and adult incisors. The epithelial component (i.e., cells that ultimately generate enamel) of developing teeth are outlined (dotted red line) with the exception of 6-week old molar where the entire tooth is outlined (I). (J,K') H&E histological

staining of control (*Stat3*$^{fl/fl}$) and mutant (*Krt14*^{Cre};*Stat3*$^{fl/fl}$) adult mice mandibular incisors showed little or no enamel matrix in mutants compared to controls.

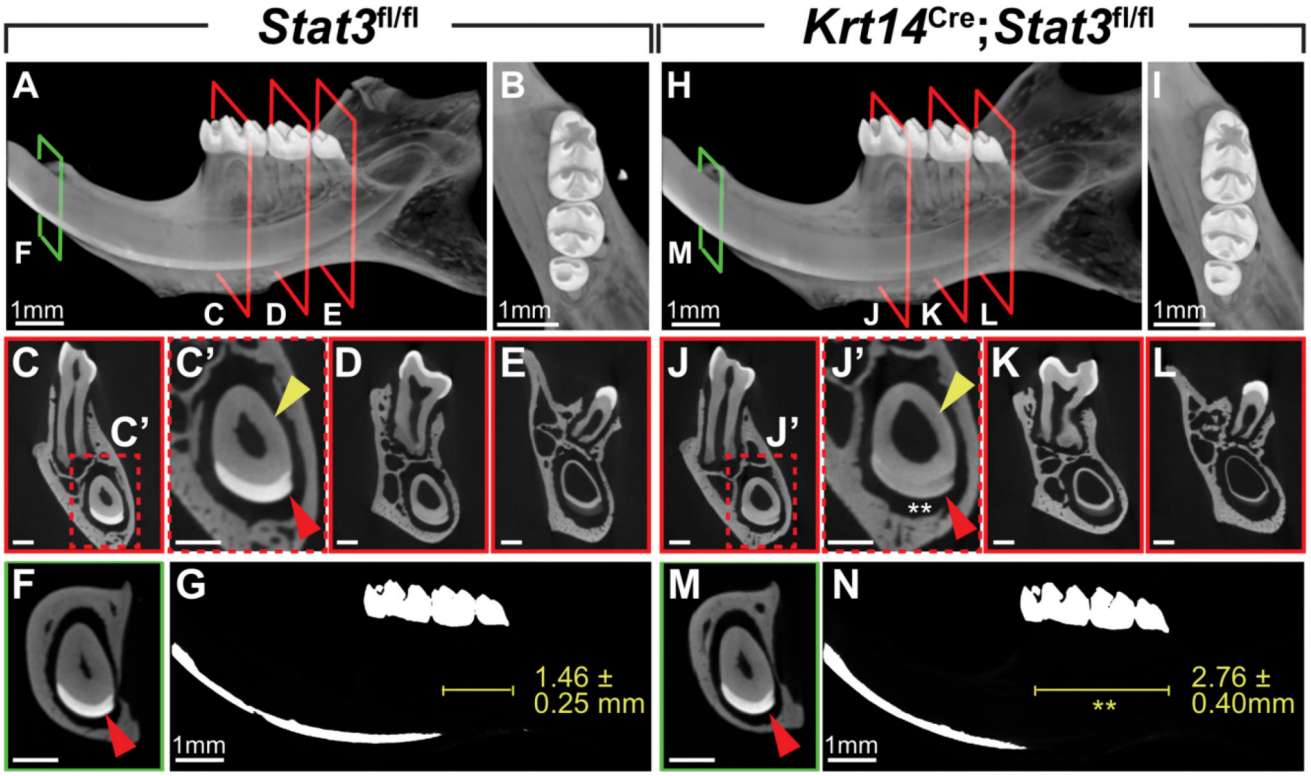

Fig. 2. Epithelial-specific inactivation of *Stat3* leads to mice with delayed incisor enamel mineralization

(A–N) MicroCT analyses of adult hemi-mandibles in sagittal view (A,H), molars in occlusal view (B,I), and the incisor in serial cross-sections (C–F and J–M). Incisor enamel is clearly mineralized underneath the 1st mandibular molar in controls (C'; red arrowhead) but not in mutants (J'; red arrowhead) and this observation was confirmed by quantifying the opacity or density of enamel (Fig. S1C–G). Dentin, the mineralized tissue abutting enamel in teeth (yellow arrowhead) appeared to be thinner in mutants (J') compared to controls (C'). Erupted enamel showed similar enamel opacities (F,M) and this observation was also confirmed by quantification (Fig. S1C,D,G). Delayed enamel mineralization in mutants (G) was clearly observed relative to controls (N) in high contrast sagittal microCT images and was quantified to be significant (Fig. S1E,F,G). **, $p<0.01$; scale bars = 50μm unless indicated otherwise.

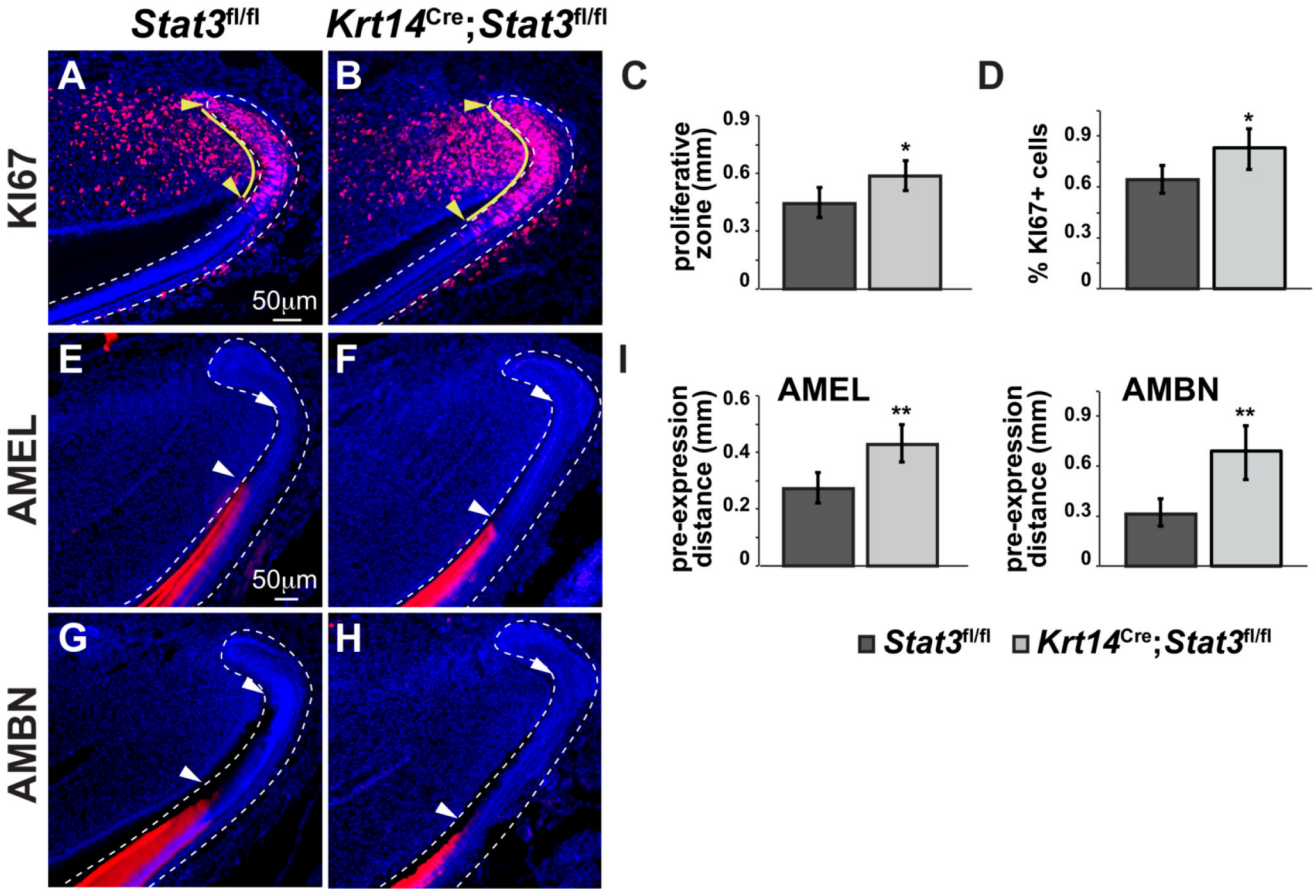

Fig. 3. Epithelial-specific inactivation of *Stat3* leads to delayed expression of AMEL and AMBN in the mouse mandibular incisor

(A,B,E–H) Immunofluorescence labeling showed KI67, amelogenin (AMEL), and ameloblastin (AMBN) expressed in control and mutant incisors. (C,D) The proliferative zone or TA region, as well as the percentage of KI67-positive cells within the laCL region, were slightly but significantly increased in mutants (region of interest is indicated by yellow line in A,B). The distance from the laCL to the start of AMEL (E,F) or AMBN (G,H) expression (i.e., pre-expression distance; distance between arrowheads) was longer in mutants compared to controls. (I) The delayed expression of AMEL and AMBN was quantified. *, $p<0.05$; **, $p<0.01$.

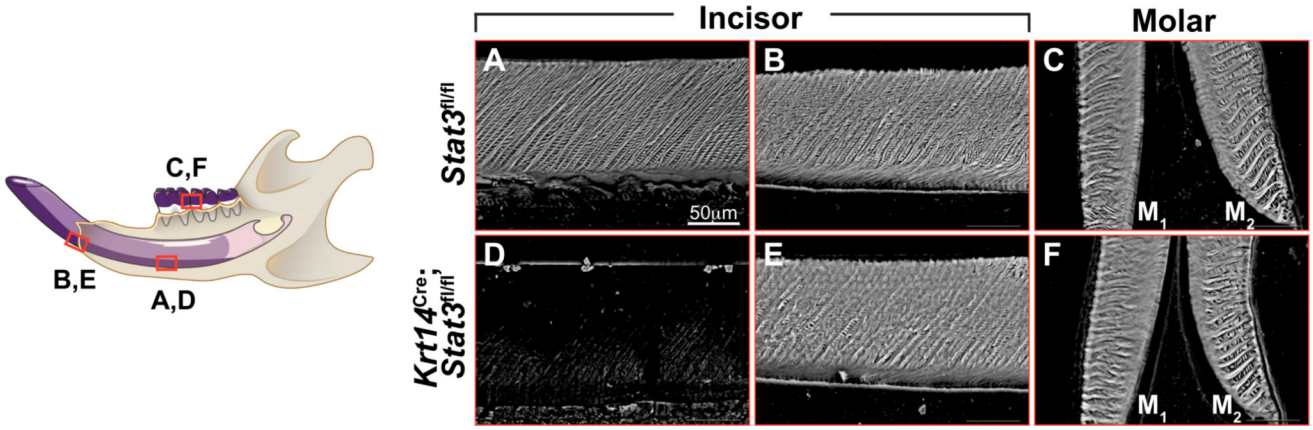

Fig. 4. Scanning electron microscopy (SEM) of mutant incisor enamel

SEM sagittal views of control and mutant incisor enamel at 3 distinct areas: (A,D) Incisor enamel at the level of the mesial cusp of the 1st mandibular molar showed differences between control and mutant incisors - control enamel mineralization appeared normal with the enamel rods extending from the DEJ to near the surface and inter-rod prisms between the enamel rods, whereas mutant enamel showed little evidence of mineralization; (B,E) The micro-architecture of the incisor enamel near the gingival margin appeared similar in control and mutant incisors; (C,F) no differences in enamel mineralization between the 1st and 2nd molars in control and mutant mice were detected.