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Juliana de Lima Gonçalves¹, Luciano Aparecido de Almeida-Júnior^{1,2}, Maya Fernanda Manfrin Arnez¹, Rodrigo Galo³, Alexandra Mussolino de Queiroz¹, Fabrício Kitazono de Carvalho¹, Francisco Wanderley Garcia de Paula-Silva^{1*}.

1. University of São Paulo, School of Dentistry of Ribeirão Preto, Developmental Biology and Child Health Research Group, Ribeirão Preto, São Paulo, Brazil.

2. Federal University of Alfenas, Alfenas, Minas Gerais, Brazil.

3. University of São Paulo, School of Dentistry of Ribeirão Preto, Department of Dental Materials and Prosthesis, Ribeirão Preto, São Paulo, Brazil.

*Corresponding author: franciscogarcia@forp.usp.br

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Ablation of *Tnfr1* gene impairs enamel surface microhardness and mineral composition in mice

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¹ University of São Paulo, School of Dentistry of Ribeirão Preto, Developmental Biology and Child Health Research Group, Ribeirão Preto, São Paulo, Brazil.

² Federal University of Alfenas, Alfenas, Minas Gerais, Brazil.

³ University of São Paulo, School of Dentistry of Ribeirão Preto, Department of Dental Materials and Prosthesis, Ribeirão Preto, São Paulo, Brazil.

***Corresponding author:** School of Dentistry of Ribeirão Preto at University of São Paulo. Avenida do Café, s/n. Bloco M, Sala 28. CEP 14040-904. Ribeirão Preto, SP, Brazil. **E-mail address:** franciscogarcia@forp.usp.br Phone: (55) 16 3315-4113.

Abstract

While the immune response is increasingly suspected to play a role in dental enamel defects, the specific pathways involved remain unclear. This study investigated the effects of *Tnfrsf1a* (TNF receptor superfamily member 1A) gene ablation on amelogenesis and enamel biomineralization. Male *Tnfrsf1a* knockout (*Tnfrsf1a*^{-/-}) and wild-type (C57BL/6) mice (n = 6 per group) were evaluated. Lower incisors were analyzed using visual photography, micro-computed tomography (microCT), scanning electron microscopy (SEM), surface microhardness testing, and energy-dispersive X-ray (EDX) spectroscopy. Protein expression of matrix metalloproteinase 20 (MMP20), RUNX family transcription factor 2 (Runx2), and ameloblastin (AMBN) was assessed via immunohistochemistry, while in situ zymography quantified MMP gelatinolytic activity. Gene expression was analyzed using RT-qPCR. *Tnfrsf1a*^{-/-} mice exhibited no macroscopic opacities, though incisors displayed a distinct whitish coloration. While microCT and SEM revealed no significant differences in enamel volume, or prism ultrastructure, the enamel of *Tnfrsf1a*^{-/-} mice showed significantly lower calcium and phosphorus content and reduced surface microhardness. Molecular analysis revealed that the *Ambn* gene was significantly upregulated during both the secretion and maturation stages in *Tnfrsf1a*^{-/-} mice compared to wild-type controls. Ablation of *Tnfrsf1a* results in enamel color change in mice characterized by altered mineral composition, reduced mechanical hardness, and dysregulated *Ambn* expression. These findings suggest that TNF signaling acts as a critical modulator during amelogenesis and is essential for achieving the final mineral quality of dental enamel.

Keywords: Biomineralization; dental enamel; *Tnfrsf1a*; tumor necrosis factor.

Introduction

Amelogenesis is the process of dental enamel formation, a complex process that occurs through different stages (Lacruz, 2017; Gil-Bona & Bidlack, 2020). In humans, dental enamel formation begins around the 12th week of gestation and is completed around 8 years of age in the permanent dentition, except for the third molars (Logan & Kronfeld, 1992). Ameloblasts are the cells responsible for dental enamel formation and undergo changes in their morphology and function according to the stage of amelogenesis (Gil-Bona & Bidlack, 2020). The secretory stage is the phase during which the organic enamel matrix is secreted by ameloblasts. At this stage, secretory granules containing minerals and proteins, such as amelogenin, ameloblastin, and enamelin, are released and guide the growth of hydroxyapatite crystals (Lacruz, 2017; Bartlett, 2013). As the process progresses, ameloblasts begin to release enzymes such as matrix metalloproteinase 20 (MMP20), which initiates the degradation of the organic content present in the tissue at this stage, thereby allowing the incorporation of minerals into the hydroxyapatite crystals. Thus, during the maturation stage, the complete removal of the organic content occurs, and, in addition to MMP20, other enzymes, such as kallikrein related peptidase 4 (KLK4), contribute to this protein removal, allowing the incorporation of calcium and phosphate ions (Bartlett et al., 2013; Lacruz, 2017; Gil-Bona & Bidlack, 2020). The absence of KLK4 can affect enamel structure such as thinner layer and decrease mineral content. The synthesis of KLK4 can be regulated by the RUNX family transcription factor 2 (Runx2). Runx2 plays an important role in bone mineralization, and mutations in this gene are related to impaired enamel formation (Chu et al., 2018).

Disturbances during the secretory and mineralization stages of enamel formation may cause developmental enamel defects (DEDs). DEDs can be classified as quantitative defects, characterized by failures during the secretion of the enamel matrix that result in hypoplasia, or qualitative defects, which occur due to failures in the mineralization process and result in hypomineralization (Salanitri & Seow, 2013; Martins, 2024). The aetiology of DEDs is considered multifactorial and can be associated with environmental, systemic and genetic factors which can be present during the pre, peri or postnatal period, yet the exact cause is still unknown (Martins, 2024; Lopes-Fatturi, 2025). Some studies have found an association between hypoxia at birth, childhood illness and the occurrence of molar-incisor hypomineralization (MIH), a common type of enamel hypomineralization (Garot et al., 2022). Occurrence of respiratory diseases systemic conditions that produce fever have been investigated as possible etiological factors of MIH, since most of these conditions start to manifest during early childhood (Bezamat, 2021; Acosta, 2022). Early childhood is an important period for enamel development, as it includes the mineralization of the permanent first molars. Systemic and environmental factors occurring during this period may potentially affect enamel formation and contribute to the development of molar-incisor hypomineralization (MIH) (Acosta, 2022). The presence of inflammatory systemic diseases during childhood leads to the production of pro-inflammatory cytokines to coordinate an immune response (Bussanelli, 2019). Several studies have shown that polymorphisms in genes involved in immune response are associated to DEDs (Pispa et al., 2004; Bussanelli, 2019; Georgina-Perez, 2023), opening the avenue for investigation of the role of inflammatory mediators and receptors in phenotypic changes in dental enamel. Recent evidence has demonstrated a direct relationship between the immune response and enamel formation (Gruper et al., 2023). Patients with APS-1, an autoimmune disease associated with mutations in the *AIRE* gene, exhibit developmental enamel defects. The *AIRE* gene plays a fundamental role in the induction of immune tolerance by

promoting the expression of tissue-specific antigens in the thymus. In this context, the authors showed that patients with APS-1 develop autoantibodies targeting proteins associated with ameloblasts and amelogenesis. Furthermore, using an *Aire* knockout animal model, it was observed structural and mineralization alterations in the enamel. These findings provide evidence that alterations in the immune response may interfere with ameloblast function and, consequently, with enamel formation and mineralization (Gruper et al., 2023).

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine that triggers multiple signaling pathways and, depending on the receptor that is activated, can play different roles in the organism (Idriss, 2000). In addition to modulating the immune and inflammatory response, TNF also plays a role in tissue repair, morphogenesis, cellular proliferation, and apoptosis (Idriss, 2000; You et al., 2021). Amelogenesis is a process dependent on the occurrence of complex cellular events, such as the presence and expression of specific proteins and enzymes required for the secretion and mineralization of the enamel matrix, in addition to cellular proliferation and apoptosis throughout the process (Gil-Bona & Bidlack, 2020; Lacruz et al., 2017). Considering that TNF is a cytokine that coordinates and regulates different cellular functions and signaling pathways, its role during enamel formation has not yet been fully elucidated. Nevertheless, some studies have already explored potential signaling pathways that may be involved in disturbances during enamel formation (Pispa et al., 2004; Ikeda et al., 2019; Nikolopoulos et al., 2020). However, the role of the TNFRSF1A signaling pathway in amelogenesis remains unknown.

Genetic studies have provided evidence that members of the TNF receptor superfamily may play important roles during enamel development (Ikeda et al., 2019; Kim et al., 2019; Nikolopoulos et al., 2020). A study using *Relt* knockout mice reported that the enamel layer had normal thickness and structure but exhibited a lower degree of mineralization, resulting in an enamel hypomineralization phenotype (Kim et al., 2019). In humans, variants in the RELT gene have been identified in patients with *amelogenesis imperfecta*, further supporting a potential role for TNF receptor superfamily members in enamel formation (Nikolopoulos et al., 2020; Ikeda et al., 2019).

The aim of this study was to evaluate the effects of *Tnfrsf1a* gene ablation, which encodes tumor necrosis factor receptor 1 (TNFR1), on enamel biomineralization using a murine model. Employing a *Tnfrsf1a* knockout model allows for the investigation of how the absence of TNFR1-mediated signaling influences enamel formation and mineralization

Materials and Methods

Ethical approval

After obtaining the approval of the Committee on Ethics in the Use of Animals at the School of Dentistry of Ribeirao Preto, University of São Paulo (Ribeirão Preto, Brazil, process n° 2019.1.139.58.0) and Internal Biosafety Committee (CIBIo, approval number #0040/98.004-01.06/2020), the animals were maintained according to the Brazilian Guideline for the Care and Use of Animals in Teaching or Scientific Research Activities regulated by the National Council for the Control of Animal Experimentation (CONCEA, Brazil, Law n° 11.794/2008). The reports were conducted in compliance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (du-Sert et al., 2020).

Animals

Six male wild-type C57BL/6 mice (*Mus musculus*) and six male mice genetically deficient in *Tnfrsf1a* (p55; 6-*Tnfrsf1a* tm1|mx) were acquired from the Central Animal Facility (Ribeirão Preto, Brazil) and the Center for the Creation of Special Mice at the School of Medicine of Ribeirão Preto, University of São Paulo (Ribeirão Preto, Brazil), respectively. Animals were 8 weeks of age, weighed 20–22 g and were kept in the Animal Facility at the School of Dentistry of Ribeirão Preto, University of São Paulo. They were housed in polypropylene cages with stainless steel lids (15 x 20 cm; 3 animals per cage), under controlled temperature (22°C $\pm$ 10%) and relative humidity (55 $\pm$ 10%), on a 12:12 h light-dark cycle, with a standard laboratory diet and free access to filtered water. For the euthanasia, the animals were first anesthetized with 10% ketamine hydrochloride (100 mg/kg; Química Farmacêutica Nacional Union Agener S/A, Embu-Guaçu, Brazil) and 2% xylazine (10 mg/kg; Dopaser, Labs Calier S/A, Barcelona, Spain), then placed in a CO₂ chamber. Then, lower and upper incisors and tissues containing bone and tooth were collected for further analysis.

Macrophotography

To analyze the presence of clinically visible changes in the enamel and subsequent comparing the groups, photographs of the animals' central incisors were taken before euthanasia. For macrophotography, a camera (Eos Rebel T2i, Canon, Tokyo, Japan) equipped with a non-polarized 100-mm f/2.8 macro lens (Canon, Tokyo, Japan) was used. The photographs were taken in labial surface of the incisors.

Scanning electron microscopy and energy dispersive X-ray analysis

After collection of the lower incisors, superficial cleaning was performed, and the teeth were kept in distilled water in a refrigerator maintained at 2–5 °C. For SEM analysis, the lower incisors were air-dried and embedded in acrylic resin. The blocks containing the teeth were sectioned transversely. The samples were submitted to surface conditioning with 37% phosphoric acid for 30 seconds, followed by washing, sonication, and air-drying for 24 hours. Then, the samples were fixed and coated with a layer of carbon. The incisors were analyzed using a JEOL-JSM-6610LV (JEOL, Tokyo, Japan) microscope at 20 kV and a working distance of 15–20 mm. For EDS analysis, the opposite lower incisors were used. Quantitative analysis of the elements was performed using the software Oxford Instruments INCA 300 EDX System (Abingdon, Oxfordshire, UK). The elements were calculated as the relative weight percentage of the total

element content (100%). The areas used for analysis were at the incisal and cervical portions. The elements quantified were calcium (Ca), phosphorus (P), carbon (C) and oxygen (O).

Knoop microhardness test

Mandibular incisors were dried and embedded in self-curing acrylic resin in the center of PVC cylinders (10 mm in diameter and 4 mm in height) with the buccal surface of the incisors positioned as close as possible to the glass plate and fixed with wax. The plastic tubes were then placed on the fragments, and acrylic resin was poured until the tube were completely filled (Stangler et al., 2013). After resin polymerization, the buccal surface of the incisors was polished with pumice and water, and microhardness analysis was performed along the surface of the incisors. One indentation was made in each of the incisal, medial, and cervical areas, with a spacing of 200 μm between each indentation, using a microdurometer (Shimadzu – HMV-2, Kyoto, Japan) equipped with a Knoop diamond tip, with a load of 10 gf for 5 seconds. Then the average of the results was calculated, and the statistical analysis was performed.

Micro-computed tomography

After the cleaning and drying procedures, the incisors were inserted into microtubes and positioned perpendicular to the radiation source. For the scanning process, a high-resolution desktop μCT system (Phoenix V tome xS240, GE, Boston, USA) was used. The scanning parameters were 70 kV and 200 μA , with a 0.1-mm Al/Cu filter, and the voxel size was 5.4 μm . The projections were acquired over a full circle of rotation at 0.4° angle intervals, and each projection consisted of the average of three transmission images. The average time of scanning was around 2 hours. The images were reconstructed using the 3D slicer software (Wayne Rasband, National Institutes of Health, USA) and then analyzed with Image J (Wayne Rasband, National Institutes of Health, USA) software. The volume of the enamel layer were evaluated in the central portion of lower incisors, next to the margin of the lower jawbone in the whole enamel layer (tip to dentin-enamel junction). For volume analysis, the images were reconstruction using 3D Slicer software (Wayne Rasband, National Institutes of Health, USA), subsequent the axial sections were reconstructed from the angular projection images using the modified Feldkamp cone-beam reconstruction algorithm, implemented in the NRecon software (v.1.7.4.2, Bruker-microCT). The total volume of the enamel layer on both incisors and molars was calculated after segmenting the enamel layer using ITK-SNAP software (Yushkevich et al., 2006).

Histological processing

The hemimandibles were dissected, removed, and fixed in 10% buffered formalin for 24 hours at room temperature and demineralized in 10% EDTA (Merck S.A. Chemical Industries, Rio de Janeiro, Brazil) for approximately 21 days. Subsequently, the samples were washed in running water for 3 hours and placed in 10% EDTA (Merck S.A. Chemical Industries, Rio de Janeiro, Brazil) for 21 days, with the EDTA solution being replaced twice weekly. On day 21, the degree of demineralization was confirmed, and the samples were washed in water for 24 hours, dehydrated in increasing concentrations of alcohol, diaphonized in xylol, and embedded in paraffin. The blocks were sectioned transversely, in an axial direction, to obtain cuts with a thickness of 5 μm .

Immunohistochemistry

The slides were deparaffinized, hydrated in a decreasing ethanol series, and kept in phosphate-buffered saline (PBS). Next, tissue sections were microwaved (7 x 12 seconds at 2-minute

intervals) with sodium citrate buffer (pH = 6.0). After temperature stabilization, the slides were washed with PBS (3x) for 5 minutes, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 40 minutes. Slides were further washed with PBS (3x) for 5 min and non-specific binding sites were blocked with 5% bovine serum albumin (Sigma-Aldrich) for 60 min. The tissues were then incubated with primary antibodies against MMP20 (orb-11059, Biorbyt, Cambridge, United Kingdom), RUNX2 (ab-23981, Abcam, USA) and AMBN (orb-155652, Biorbyt, Cambridge, United Kingdom) at 4°C overnight. Next, slides were washed and incubated with a mouse anti-rabbit (sc-2491, Santa Cruz Biotechnology, Dallas, Texas, USA) biotinylated secondary antibody for one hour. Then, the slides were washed in PBS and incubated with streptavidin conjugated with horseradish peroxidase (HRP) for 20 minutes. 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, Missouri, USA) was used as the enzyme substrate for five minutes; the slides were washed with PBS, counterstained with hematoxylin for 15 seconds, washed with distilled water, dehydrated in increasing ethanol concentrations, and mounted in Entellan® (Merck, Darmstadt, Germany). Negative control slides, in which the primary antibody was omitted, were used to test the specificity of immunostaining. Then, images of the histological sections from each group and for each marker were obtained at 20× and 40× magnifications using an AxioLab5 microscope (Carl Zeiss, Oberkochen, Germany), capturing the entire ameloblast layer to demonstrate the expression of these proteins.

In situ zymography

In situ zymography was carried out in order to detect gelatinolytic activity in the ameloblast layer. The slides were initially prepared by dehydration and diaphanization, as described above. Subsequently, they were added to sodium borohydride solution (1 mg/mL) (3x) for 15 minutes, followed by incubation with FITC and gelatin substrate (DQTM Gelatin, Molecular Probes, Eugene, Oregon, USA) dissolved in agarose (0.1 mg/mL) for two hours at 37 °C in an oven protected from light. Nuclear staining was carried out by adding DAPI (0.5 µg/mL) to the sections. As a negative control, slides were incubated with ethylenediamine tetraacetic acid (EDTA) (EDTA, Sigma, St. Louis, Missouri, USA) for 1 hour and then incubated with gelatin substrate and EDTA. The analysis of gelatinolytic activity in the sections was performed using Image J software by quantifying the fluorescent intensity in the ameloblast layers at 10× magnification under the microscope and expressing the number of enzymatic activity points per mm². For the analysis, the image taken with the FITC filter was opened and converted to black and white. After that, the ameloblast layer was delimited and measured. The values collected were the integrated density and area, and the ratio between these values was used for the statistical analysis.

Evaluation of gene expression by reverse transcription and real-time polymerase chain reaction

The enamel organs of the mandibular and maxillary incisors were dissected from wild-type (n=6) and *Tnfrsf1a*^{-/-} (n=6) mice. Tissues from secretory and maturation stages were stored in RNAlater solution (Invitrogen, Thermo Fisher Scientific, Massachusetts, EUA) and kept at -80 °C. Total RNA was extracted from secretion and maturation samples using the PureLink™ kit (Invitrogen, Thermo Fisher Scientific, Massachusetts, EUA) according to the manufacturer's instructions. Total RNA was quantified through UV absorbance using a NanoDrop (Thermo Fisher Scientific, Massachusetts, EUA). RNA sample quality was evaluated using the OD260/280 ratio, and values between 1.8 and 2.0 were considered indicative of good RNA quality. cDNA was synthesized by means of a reverse transcription reaction, starting with 1 µg of total RNA (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). The primers selected were

Ambn (*Ambn*, Mm00477486_m1), matrix metalloproteinase 20 (*Mmp20*, Mm00600244_m1) and RUNX family transcription factor 2 (*Runx2*, Mm00501584_m1). The genes encoding for the enzyme glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, Mm9999991561_g1) and for beta-actin (*Actb*, 02619580_g1) were used as reference. Amplification was performed under the following conditions: activation of TaqMan Fast Advanced Master Mix polymerase (Applied Biosystems, Thermo Fisher Scientific Inc) at 95 °C for two seconds, followed by 40 cycles at 95°C for 1 second for DNA denaturation and at 60 °C for 20 seconds for primer annealing and polymerization. Relative quantification of gene expression was performed using the $\Delta\Delta C_t$ method.

Statistical analysis

The data obtained from each analysis were tabulated using GraphPad Prism 10.0 (Dotmatics, San Diego, USA) software. First, the data was subjected to the Shapiro-Wilk normality test. All data showed a normal distribution, so parametric Student's *t*-test was used to compare the two groups. The significance level was set at 5%.

Results

Macroscopic evaluation and scanning electron microscopy (SEM)

In the visual inspection analysis of the lower incisors, it was possible to observe macroscopic differences in the enamel layer between wild-type and *Tnfrsf1a*^{-/-} mice. Although the enamel layer was intact and showed no opacities, the *Tnfrsf1a*^{-/-} animals exhibited incisors with a whitish coloration and less pigmented yellow coloration which represents the iron deposition that occurs during the maturation stage of amelogenesis (**Fig. 1**).

In the SEM images, there were no differences in the enamel structure between the two groups. In wild-type animals, a lattice pattern and a well-arranged layer of enamel rods were observed, which were also present in the enamel layer of *Tnfrsf1a*^{-/-} animals (**Fig. 2**).

Energy-dispersive X-ray spectroscopy, Knoop microhardness and dental enamel volume

Dental enamel from wild-type mice showed a higher content of calcium ($p = 0.002$), phosphorus ($p = 0.034$) and a lower content of carbon ($p = 0.002$) compared with *Tnfrsf1a*^{-/-} mice. The oxygen content showed no difference between the groups ($p = 0.285$). Enamel microhardness was significantly higher in wild-type animals than in the *Tnfrsf1a*^{-/-} animals ($p = 0.003$).

Enamel layer volume was reduced in knockout animals ($p > 0.0043$) when compared to knockout mice (**Table 1**) (**Fig. 3**).

Animal	Ca	P	O	C	Microhardness	Volume (mm ³)
Wild-type	40.80 ± 3.4*	14 ± 2.7*	18.78 ± 5.75	24.03 ± 6.16*	250.5 ± 35.86*	0.79 ± 0.07*
<i>Tnfr1</i> ^{-/-}	26.49 ± 3.96	9.09 ± 2.63	23.3 ± 5.43	41.80 ± 9.69	153.5 ± 29.26	0.52 ± 0.04

Table 1. Chemical elements (%/w) found in dental enamel, Knoop microhardness (KHN) test results and dental enamel volume in mm³. *Indicates a statistically significant difference between the groups. Ca = calcium, P = phosphorus, O = oxygen; C = carbon.

Amelogenesis signaling during enamel biomineralization

In wild-type animals, *Mmp20* mRNA expression showed no differences between the secretory ($p = 0.062$) and maturation stages ($p = 0.749$) when compared with *Tnfrsf1a*^{-/-} mice (**Fig. 4**). Immunohistochemistry revealed a different expression pattern between the groups. Wild-type animals showed low and homogeneous MMP20 expression throughout the ameloblastic layer and enamel organic matrix, whereas *Tnfrsf1a*^{-/-} animals presented a different staining pattern (**Fig. 4**).

In addition, the gelatinolytic activity of metalloproteinases in *Tnfrsf1a*^{-/-} animals was higher in the ameloblastic layer ($p = 0.031$) and in the enamel organic matrix ($p = 0.023$) compared with wild-type animals (**Fig. 5**).

The RUNX family transcription factor 2 had low expression in the ameloblast layer, which was similar between wild-type and *Tnfrsf1a*^{-/-} mice. The *Runx2* mRNA expression in the secretory

stage was similar between knockout and wild-type animals ($p = 0.058$). At the maturation stage, the expression did not differ between the groups ($p = 0.924$) (**Fig. 6**).

Regarding ameloblastin, *Tnfrsf1a*^{-/-} animals showed a high staining intensity in ameloblast layer with similar staining in the enamel organic matrix compared with wild-type mice. The expression of *Ambn* mRNA was upregulated in both secretory ($p = 0.010$) and maturation stages ($p = 0.015$) of *Tnfrsf1a*^{-/-} mice when compared to wild-type mice (**Fig. 7**).

Discussion

The present study aimed to describe the effects that the absence of the pro-inflammatory TNF receptor superfamily member 1A could have on enamel biomineralization in mice. It was found that the absence of the receptor can affect amelogenesis, leading to macroscopic changes in the enamel surface and mineral content. In addition, it was observed that *Ambn* gene expression was upregulated during the secretory and maturation stages of amelogenesis, stages in which ameloblastin plays an important role in enamel formation, and alterations in its gene expression could affect enamel development. (Lacruz et al., 2017; Bartlet, 2013).

The present results showed that although there were no visual opacities in the dental enamel, the knockout mice exhibited a whitish coloration when compared to wild-type animals. This could be explained by possible alterations in iron deposition on the labial side of mouse incisors (Wen et al., 2013). During the maturation stage, ameloblasts incorporate iron which is then deposited beneath the mature layer of enamel (Stein & Boyle, 1958). The function of iron in the enamel surface is not well known (Stein & Boyle, 1958; Fan et al., 2022). Although previous studies showed that iron is directly related to incisor pigmentation, recent evidence demonstrated that iron contributes to the formation of an acid-resistant surface layer, which the authors called Fe-rich enamel (Fe-EN) (Srot et al., 2024). The study suggests that iron is incorporated into dental enamel as ferrihydrite-like deposits, which are positioned between the hydroxyapatite crystals, forming the Fe-EN. Furthermore, the authors showed that the yellowish coloration found in the incisors is related to the thickness and structural organization of the surface layer, which generates light transmission through the Fe-EN, and not directly to the amount of iron that was incorporated into the enamel (Srot et al., 2024). The role of TNF has been described in iron metabolism in other cell types such as monocytes and endothelial cells (Scaccabarozzi et al., 2000, Nanami et al., 2005). Iron regulation in tissues and cells can be mediated by specific proteins such as transferrin receptor (TFRC) and divalent metal transporter 1 (DMT1) (Nanami et al., 2005). An increase in TNF levels due to inflammatory processes enhances the activity of these proteins, consequently leading to increased iron influx into endothelial cells (Nanami et al., 2005). However, this study did not explore iron content or metabolism during amelogenesis, but the whitish coloration observed in mice incisors suggests that iron deposition could be impacted in the absence of *Tnfrsf1a*.

Regarding enamel ultrastructure, SEM analysis did not reveal any alterations in the pattern of enamel rods in knockout mice. Changes in the ultrastructure of the enamel layer were reported by Liang et al. (2021), who described the presence of dental enamel defects in mice with a mutation in dentin sialophosphoprotein. The superficial enamel layer of transgenic mice showed a loss of the rod pattern and a reduction in enamel volume indicating that enamel maturation was delayed, which were suggested to be associated with the intracellular retention of mutant DSPP in presecretory ameloblasts (Liang et al., 2021). Furthermore, the role of ameloblastin was investigated in the formation and organization of enamel prisms during amelogenesis using genetically modified mouse models (Lu et al., 2011). In the SEM analysis results, it was observed that hydroxyapatite crystals in wild-type animals were long and parallel to each other, and the same pattern was observed in mice overexpressing *Ambn* (Lu et al., 2011). These findings suggest that AMBN overexpression alone is not sufficient to significantly influence the growth and orientation of hydroxyapatite crystals. However, transgenic mice overexpressing ameloblastin in the absence of amelogenin showed shorter and randomly oriented crystals, suggesting that

interactions among proteins in the enamel organic matrix play an important role in crystal organization and growth. In the present study, *Tnfrsf1a*^{-/-} mice exhibited increased *Ambn* mRNA expression and no alterations in the organization pattern of the enamel prisms observed in SEM. Previous study investigated the role of RELT, a receptor belonging to the tumor necrosis factor receptor superfamily, in enamel formation and showed that incisors of *Relt* knockout mice exhibited a less translucent appearance (Kim et al., 2019). SEM analysis showed that the enamel layer appeared chalkier and had a rougher surface (Kim et al., 2019). Although the exact role of RELT is still being studied, it has been suggested that the receptor may operate through a mechanism similar to that of mutations in the *Lamb3* gene, which cause failures in ameloblast attachment to the enamel matrix (Nikolopoulos et al., 2020; Smith et al., 2019).

The absence of *Tnfrsf1a* influence the volume of the enamel layer and it was also possible to observe a decrease in microhardness and in the calcium and phosphorus content in enamel. The transport of Ca²⁺ to hydroxyapatite crystals during amelogenesis is mediated by specific ion channels in ameloblasts such as PMCA-1 (Gonçalves et al., 2024; Nurbaeva et al., 2016). Several studies have demonstrated that TNF plays a regulatory role in various ion channels in cardiomyocytes, macrophages, and odontoblast-like cells (Yarilina et al., 2011; El Karim et al., 2015; Rao et al., 2016). El Karim et al. (2015) provided evidence that TNF, which is highly expressed in dentin during inflammatory processes, can activate TRPA1 and TRPV4 channels in odontoblasts. TRP channels are a class of ion channels expressed in various tissues and cell types, responsible for regulating the influx of ions such as Ca²⁺, Mg²⁺, Na⁺, and K⁺ (Zhang et al., 2023). The expression of TRPC1 and TRPC7 in rat ameloblasts has already been reported (Yang et al., 2017). Considering the interaction between TRPA1/TRPV4 channels and TNF in odontoblasts, it is plausible to investigate whether TNF could play a role in the regulation of TRPC1 and TRPC7 channels in ameloblasts, since the present study revealed a decrease in enamel calcium and phosphorus content in *Tnfrsf1a* knockout animals.

In addition, an increased protein content has been reported in the enamel layer of human teeth with DEDs (Farah et al., 2010), although this was not investigated in this study. In the present study *Mmp20* gene expression was similar in the secretory and maturation stages in both groups of mice. *Ambn* expression was upregulated in both the secretory and maturation stages. MMP20 is an enzyme that plays a role during the secretory phase and the early maturation phase. The main role of MMP20 is to remove proteins that are present in the enamel organic matrix, allowing mineral incorporation and extension of hydroxyapatite crystals (Bartlett et al., 2006; Bartlett, 2013; Prajapati et al., 2016). The absence of MMP20 in the teeth of rodents revealed that those teeth had low mineral content, as MMP20 plays a role in the removal of proteins such as amelogenin and guides the growth in length and thickness of hydroxyapatite crystals (Bartlett, 2013). Studies have shown that in most cases where there are molecular changes in the signaling of enamel formation, there is a failure in the removal of proteins from the enamel organic matrix, which leads to a reduction in mineral content and an increase in the organic component (Bartlett, 2013; Mu et al., 2022). Bussanelli et al. (2019) explored the interaction between genes involved in the immune response and amelogenesis. A single nucleotide polymorphism (SNP) in the AMELX gene was reported to interact with genes related to the inflammatory response, such as IL10 and IL17A. The AMELX gene encodes amelogenin, a key protein of the organic enamel matrix, essential for enamel formation. Although the authors did not observe an interaction with the TNF gene (Bussanelli et al., 2019), the present study suggests that the absence of the receptor for this cytokine

in mice can affect the expression of essential proteins such as ameloblastin during amelogenesis and should be further investigated.

Runx2 plays an important role in various stages of osteogenesis, and also during odontogenesis and amelogenesis (Camilleri et al., 2006). The absence of *Runx2* during amelogenesis results in a high protein content in the organic matrix, primarily ameloblastin, amelogenin, and enamelin, demonstrating that Runx2 is essential during enamel formation (Chu et al., 2021). The presence of TNF in cells, such as osteoblasts, has been shown to decrease or inhibit the expression of Runx2 (Gilbert et al., 2002; Kaneki et al., 2006). In the present study, the gene expression of *Runx2* during the secretory and maturation stages of amelogenesis was similar between groups. This finding suggests that the absence of *Tnfrsf1a* did not affect *Runx2* expression in ameloblasts, although studies suggest that TNF has a role in the regulation of Runx2 expression (Gilbert et al., 2002; Kaneki et al., 2006). Overexpression of this transcription factor has previously been associated with altered enamel mineralization, including reduced expression of KLK4, a protease responsible for protein removal during the maturation stage (Mu et al., 2022).

This study was limited to male animals, which may affect the generalizability of the findings. There are known differences in metabolic and signaling pathways between male and female animals, which may affect the extrapolation of study results (Orsini et al., 2017). The possible effects of a pro-inflammatory cytokine were explored, and previous research has shown that female animals can exhibit different inflammatory responses and cytokine expression compared with male mice (Crisostomo et al., 2007). For this reason, general assumptions should not be made, and further investigation is needed.

The pro-inflammatory receptor TNFRSF1A may act as a modulator of amelogenesis, since its absence in male mice was associated with whitish color change, reflected by subtle changes in enamel mineral composition and hardness. In addition, altered gene expression of *Ambn* was observed; this may influence the enamel formation process and potentially contribute to the occurrence of dental enamel defects.

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Data availability statement

Data that support the findings of this study are available from the corresponding author, FWGPS, upon reasonable request.

Author Contribution Statement

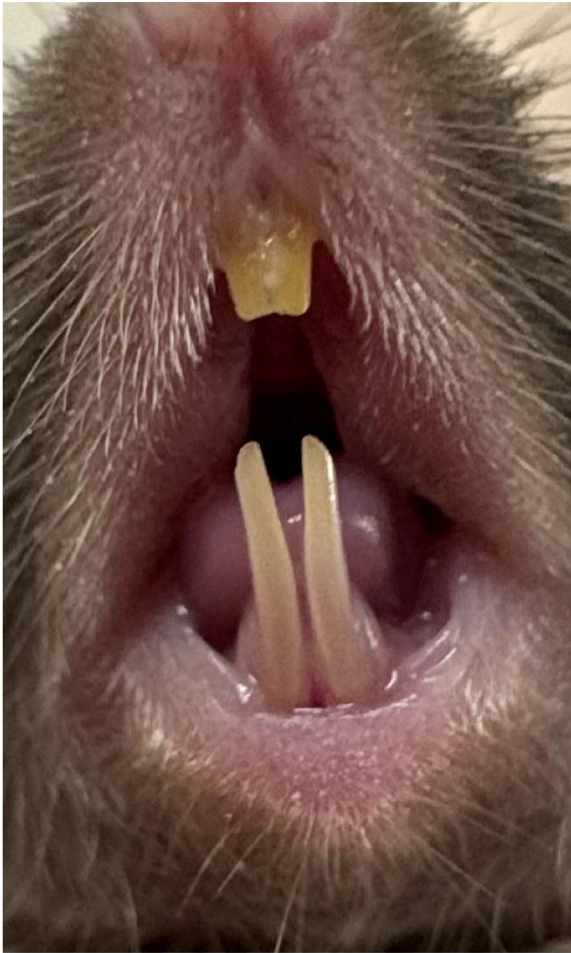
Conceptualization: Paula-Silva, FWG; Formal analysis: Paula-Silva FWG, Gonçalves JL; Investigation: Gonçalves JL, Almeida-Júnior LA; Methodology: Paula-Silva FWG, Gonçalves JL, Almeida-Júnior LA, Galo R; Writing – original draft: Gonçalves JL; Writing – review & editing: Paula-Silva FWG, Arnez MFM, Queiroz AM, Carvalho FK.

Declaration of Competing Interests

The authors declare no conflicts of interests.

FIGURE LEGENDS

Wild-type



Tnfrsf1a^{-/-}

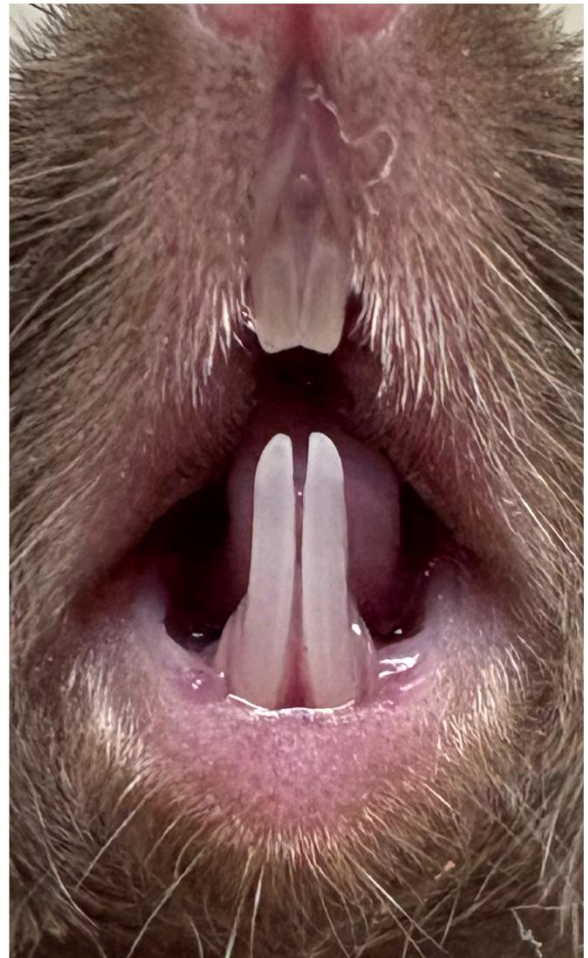


Fig. 1. The mandibular incisors of wild-type animals exhibited a yellowish coloration, meanwhile *Tnfrsf1a*^{-/-} mice exhibited a whiter appearance.

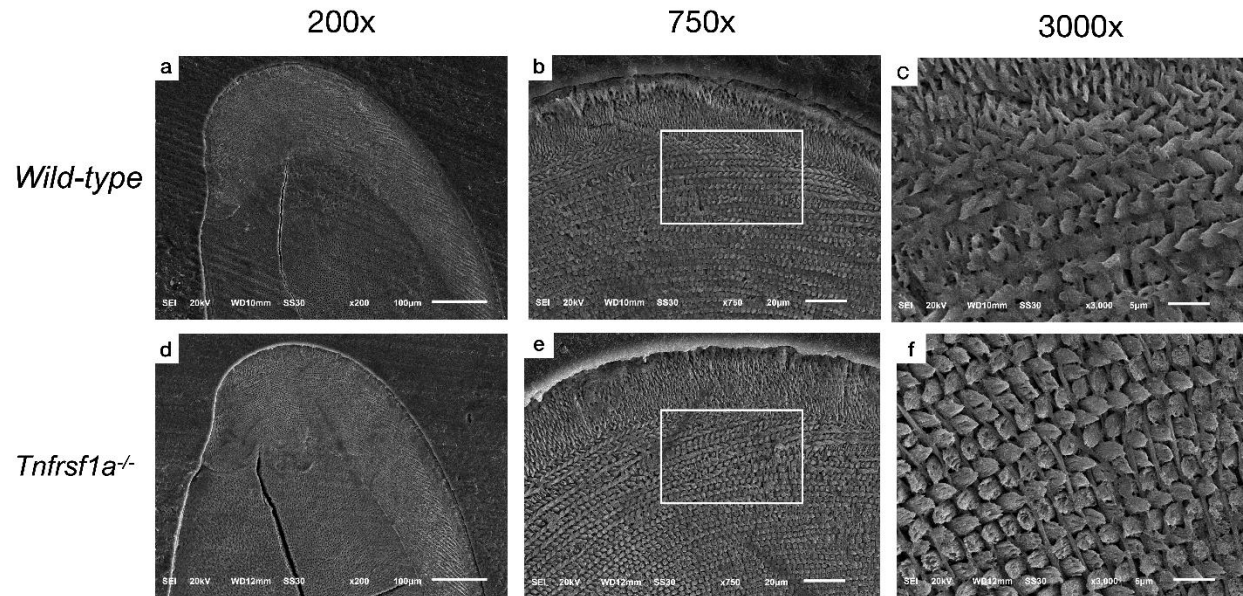


Fig. 2. Transverse sections of the dental enamel layer from lower incisors after conditioning with 37% phosphoric acid in wild-type animals (a, b, c) and *Tnfrsf1a*^{-/-} animals (d, e, f). Scale bar (a, d) 100μm; (b, e) 20μm; (c, e) 5μm.

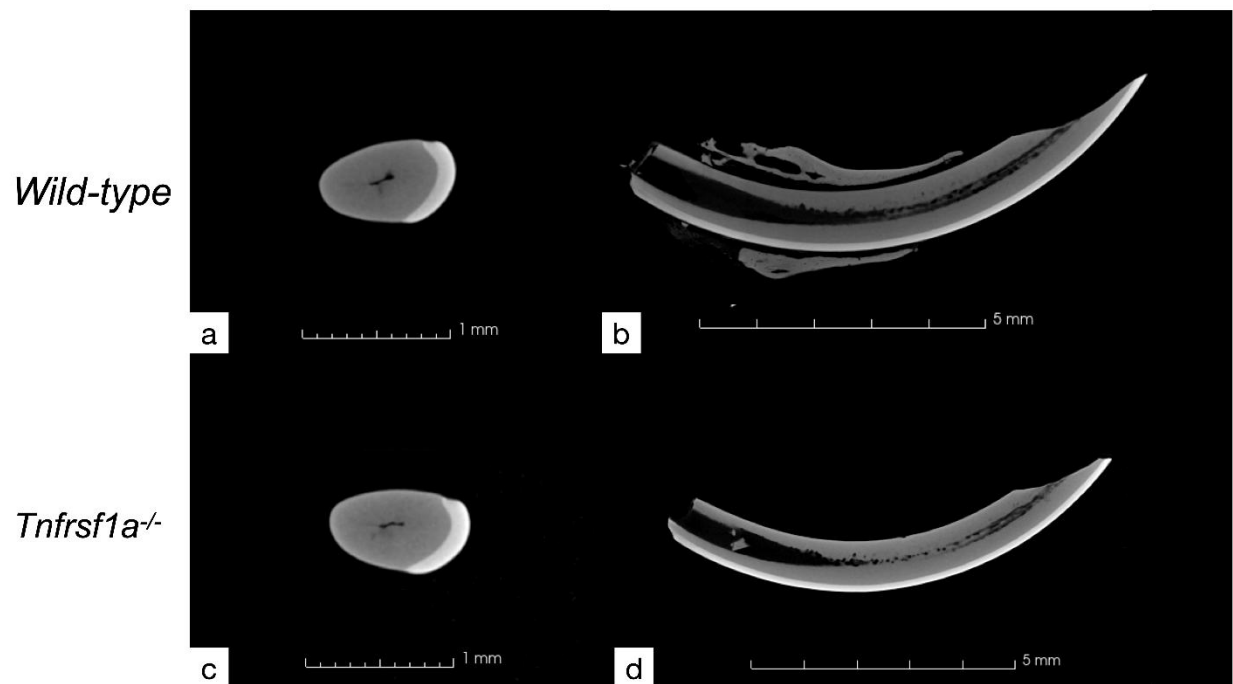


Fig. 3. Nanotomography (μCT) analysis of the enamel of lower incisors from wild-type and *Tnfrsf1a*^{-/-} mice. Axial sections (a) and (c) (scale bar, 1 mm); and sagittal sections (b) and (d) (scale bar, 5 mm) of the incisors showing the mineralized enamel layer.

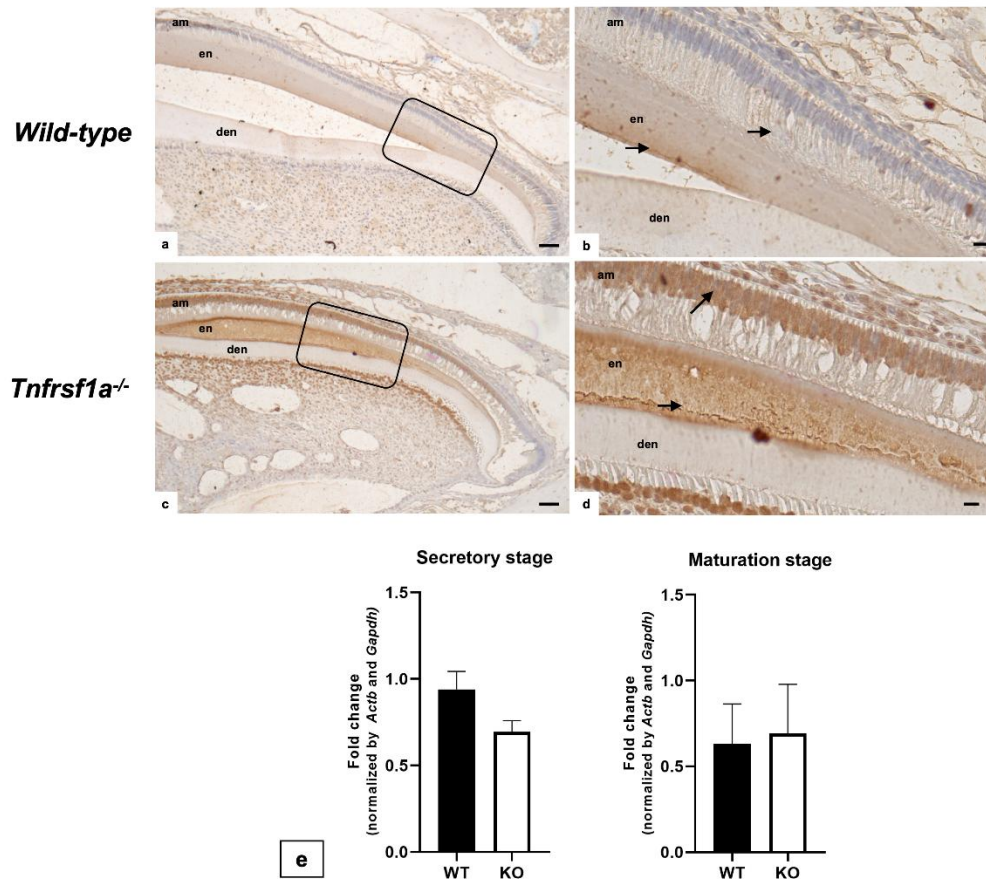


Fig. 4. Matrix metalloproteinase 20 (MMP20) immunostaining and *Mmp20* mRNA expression in wild-type and *Tnfrsf1a* mice. Immunostaining in wild-type (a, b) and knockout (c, d) animals; (e) *Mmp20* mRNA levels during the secretory and maturation stages of amelogenesis. Error bars in the graphs indicate the standard deviation. Am, ameloblasts; En, enamel layer; Den, dentin. Scale bars: (a, c) 100 μ m; (b, d), 20 μ m.

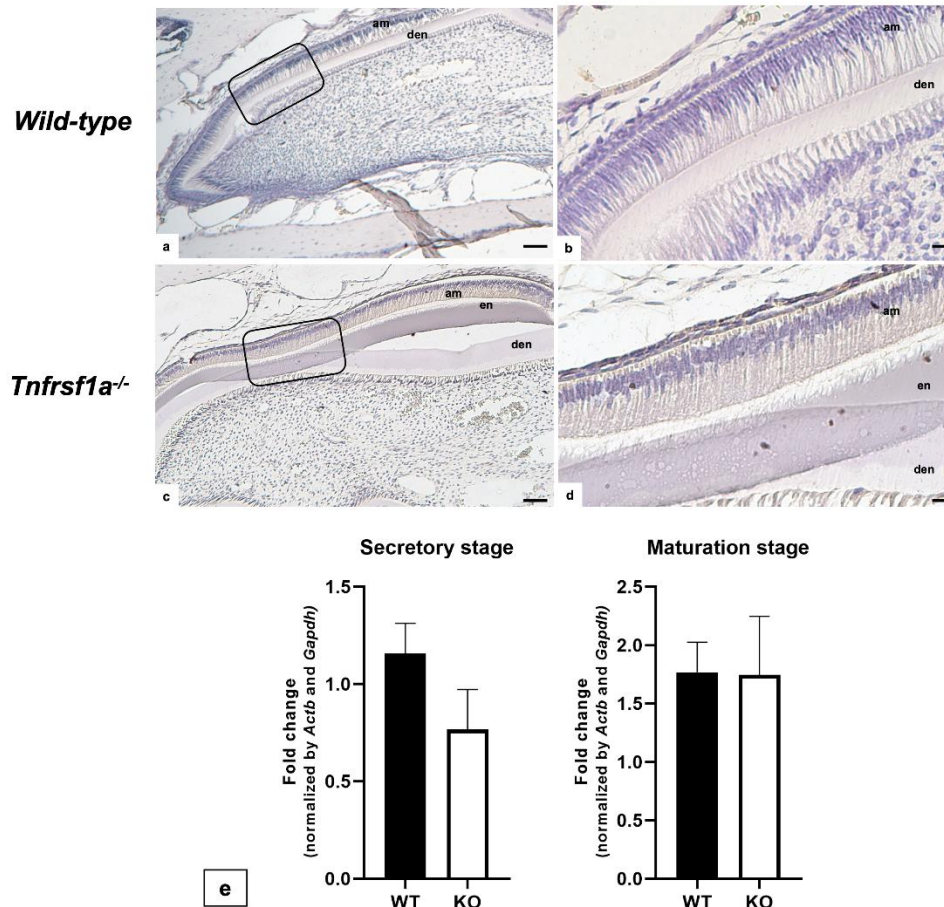


Fig. 6. RUNX family transcription factor 2 immunostaining in ameloblast layer and *Runx2* mRNA expression in wild-type and *Tnfrsf1a*^{-/-} mice. Images showing RUNX2 expression in wild-type (a, b) and knockout (c, d) animal. *Runx2* mRNA levels during the secretory and maturation stages in both groups (e). Am, ameloblasts; En, enamel layer; Den, dentin. Error bars in the graphs indicate the standard deviation. Scale bars: (a, c) 100 μ m and (b, d) 20 μ m.

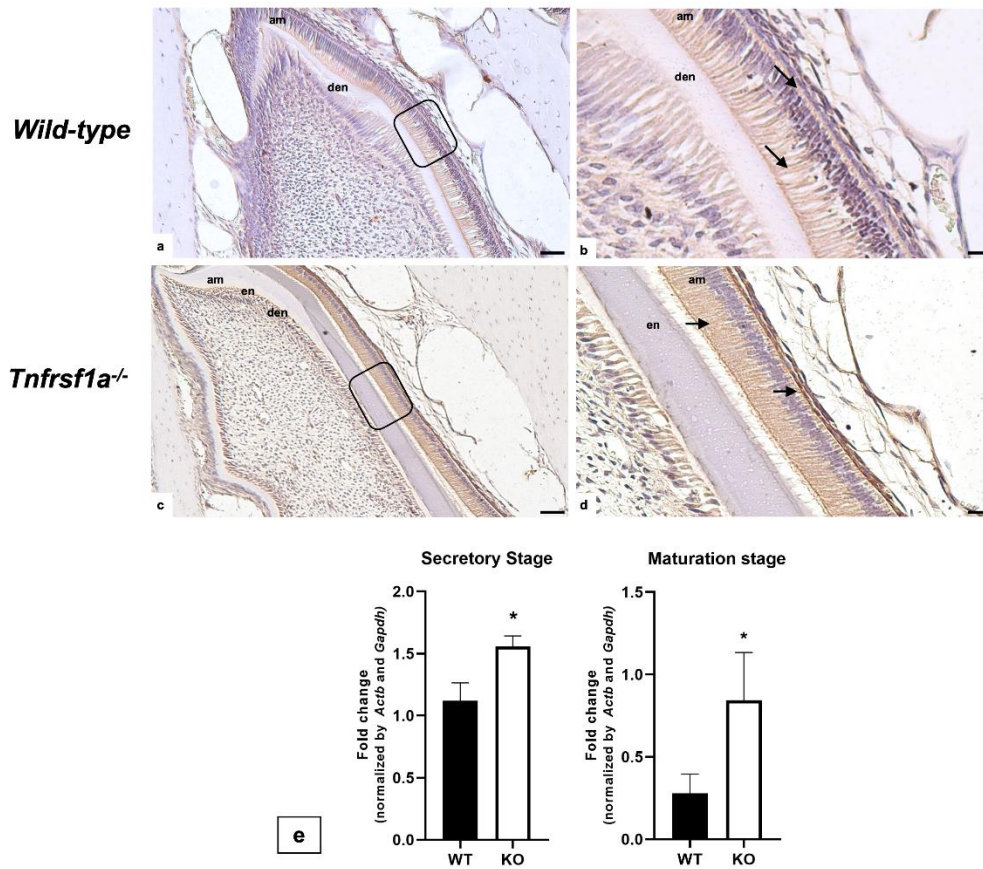


Fig. 7. AMBN expression in the ameloblast layer of knockout and wild-type animals. Immunostaining in wild-type (**a**, **b**) and knockout (**c**, **d**) animals. mRNA expression of *Ambn* was upregulated in the secretory and maturation stages of knockout mice compared with wild-type mice (**e**). Am, ameloblasts; En, enamel layer; Den, dentin. Error bars in the graphs indicate the standard deviation. * indicates $p < 0.05$. Scale bars: (**a**, **c**) 100 μm, (**b**, **d**) 20 μm.