



# Deficiency of Mineralization-Regulating Transcription Factor *Trps1* Compromises Quality of Dental Tissues and Increases Susceptibility to Dental Caries

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Dental caries is the most common chronic disease in children and adults worldwide. The complex etiology of dental caries includes environmental factors as well as host genetics, which together contribute to inter-individual variation in susceptibility. The goal of this study was to provide insights into the molecular pathology underlying increased predisposition to dental caries in trichorhinophalangeal syndrome (TRPS). This rare inherited skeletal dysplasia is caused by mutations in the *TRPS1* gene coding for the TRPS1 transcription factor. Considering *Trps1* expression in odontoblasts, where *Trps1* supports expression of multiple mineralization-related genes, we focused on determining the consequences of odontoblast-specific *Trps1* deficiency on the quality of dental tissues. We generated a conditional *Trps1<sup>Col1a1</sup>* knockout mouse, in which *Trps1* is deleted in differentiated odontoblasts using *2.3kbCol1a1-Cre<sup>ERT2</sup>* driver. Mandibular first molars of 4wk old male and female mice were analyzed by micro-computed tomography ( $\mu$ CT) and histology. Mechanical properties of dentin and enamel were analyzed by Vickers microhardness test. The susceptibility to acid demineralization was compared between WT and *Trps1<sup>Col1a1</sup>cKO* molars using an *ex vivo* artificial caries procedure.  $\mu$ CT analyses demonstrated that odontoblast-specific deletion of *Trps1* results in decreased dentin volume in male and female mice, while no significant differences were detected in dentin mineral density. However, histology revealed a wider predentin layer and the presence of globular dentin, which are indicative of disturbed mineralization. The secondary effect on enamel was also detected, with both dentin and enamel of *Trps1<sup>Col1a1</sup>cKO* mice being more susceptible to demineralization than WT tissues. The quality of dental tissues was particularly impaired in molar pits, which are sites highly susceptible to dental caries in human teeth. Interestingly, *Trps1<sup>Col1a1</sup>cKO* males demonstrated a stronger phenotype than females, which calls for attention to

genetically-driven sex differences in predisposition to dental caries. In conclusion, the analyses of *Trps1*<sup>Col1a1</sup>cKO mice suggest that compromised quality of dental tissues contributes to the high prevalence of dental caries in TRPS patients. Furthermore, our results suggest that TRPS patients will benefit particularly from improved dental caries prevention strategies tailored for individuals genetically predisposed due to developmental defects in tooth mineralization.

**Keywords:** odontoblasts, mineralization, conditional knockout mice, dental caries, trichorhinophalangeal syndrome

## INTRODUCTION

Dental caries remains the most prevalent chronic disease affecting 60–90% of children and adolescents (5–17 years of age), and nearly every adult among all populations (1–4). This complex multifactorial disease involves progressive destruction or demineralization of teeth, caused by acids produced by commensal bacteria within oral biofilms (5–7). Initially, dental caries affects only enamel. As the caries lesion progresses, crown dentin becomes affected. Cementum and root dentin are only affected when roots of teeth are exposed to the oral cavity, which occurs mostly in elderly patients (8). Of note, individual tooth surfaces have vastly different susceptibilities to caries, with the occlusal pits and fissures of molars being the most susceptible (9, 10).

Many environmental, endogenous, and behavioral risk factors have been identified as contributors to the development of dental caries (4, 11, 12). Nevertheless, evidence from genome-wide association studies (GWAS), complex segregation analysis and twin as well as family-based studies indicate that susceptibility to dental caries is strongly influenced by each individual's genetic constitution, which dictates tooth anatomy (e.g., the depth of pits and fissures), quality of dental mineralized tissues, salivary properties, host immunity and taste preference (13–18). One potential mechanism of a genetic contribution to dental caries is the formation of hypomineralized dental tissues that are more prone to acid-caused destruction (19). This is, for example, the case in genetic diseases such as dentinogenesis imperfecta and hypophosphatasia, providing evidence that high susceptibility to dental caries initiation and progression is associated with decreased tooth mineral content (19, 20).

Mineralization of dental tissues depends on multiple factors, including protein composition of the extracellular matrix (ECM), post-translational modifications of ECM proteins, availability of calcium (Ca<sup>2+</sup>) and phosphate (P<sub>i</sub>) ions, pH, and the presence of mineralization inhibitors (21–23). Additionally, proper transcriptional regulation of the function of cells producing mineralized ECM is crucial for formation of sound mineralized tissues (24–26). This has been well demonstrated for two major mineralization-supporting transcription factors: Runx2 and Osx/Sp7. Specifically, a significant reduction in mineralization and size of enamel rods was reported in *Runx2* conditional knockout (cKO) mice (26), while studies of *Osx/Sp7* null mice revealed disrupted differentiation of both odontoblasts and ameloblasts, affecting tooth mineralization

(25). Although, these *in vivo* studies highlighted the importance of transcriptional regulation in the formation of dental tissues, the contribution of mineralization-regulating transcription factors in the predisposition to dental caries has yet to be elucidated, in spite of evidence from GWAS studies identifying genes coding for transcription factors as dental caries risk loci (27, 28).

*Trps1* is a transcription factor involved in tooth development and mineralization of skeletal and dental tissues (29–34). *Trps1* is encoded by the *Trps1* gene located on the distal half of the chromosome 15 in the mouse genome, and on the long arm of the chromosome 8, at 8q24, in the human genome (31). The name of this protein and the gene come from the trichorhinophalangeal syndrome (TRPS), a rare autosomal-dominant skeletal dysplasia caused by mutations in the *TRPS1* gene (31). Moreover, the name of this condition refers to some of the most characteristic features observed in affected individuals: tricho—derives from the Greek “trikhos”, meaning “hair”; rhino—comes from the Greek “rhís”, meaning “nose”; while phalangeal—refers collectively to the digital bones in the hands and feet. Patients with this syndrome have fine, sparse, and slow-growing scalp hair, a characteristic bulbous nose with a broad nasal septum and tip, and short digits with radiographically visible cone-shaped epiphyses restricted to the middle or proximal phalangeal bones (33, 35). In addition to epiphyseal defects, characteristic to TRPS is short stature, hip dysplasia and premature closure of growth plates (31, 35), all indicative of defective development of endochondral bones. TRPS patients also present with hypoplastic mandibular condyles, reduced bone mineral density (osteopenia), and midface hypoplasia (32, 36, 37). Severe osteoporosis has been reported in some affected individuals (38).

The array and severity of phenotypic manifestations of TRPS differ among affected individuals and even within family members (33, 35). There is, however, some genotype-phenotype correlation, which allowed to distinguish three types of TRPS. TRPS type I (OMIM#190350) is a milder form, caused by nonsense mutations resulting in *TRPS1* haploinsufficiency. More severe TRPS type III (OMIM#190351) is caused by missense mutations located exclusively in the exon 6 and 7 of *TRPS1* gene, resulting in functional modification of the DNA binding domain of the TRPS1 transcription factor (35, 39). These mutations are predicted to have a combination of a loss- and gain-of-function effect. TRPS type II or Langer-Giedion syndrome (OMIM#150230) is caused by a contiguous gene deletion,

which, in addition to *TRPS1*, also encompasses the adjacent gene for multiple hereditary exostoses *EXT1* (40, 41). Hence, the phenotype of type II combines features of TRPS type I with multiple exostosis, while individuals with TRPS type III demonstrate severe brachydactyly and short stature (42, 43).

TRPS is one of a few genetic syndromes with frequently reported dental abnormalities, although most of the reports only briefly mention dental phenotypes, without their detailed characterization. Nonetheless, TRPS oral findings reported in (sparse) literature encompass defects in tooth number, size, shape, and mineralization. The most common TRPS dental abnormalities include supernumerarity, microdontia, malocclusion, and delay in root and crown development. Cases of hypodontia, abnormal tooth morphogenesis, impaired dentin mineralization, large dental pulp chambers, and extensive dental caries have also been reported, underscoring the clinical importance of *TRPS1* for tooth development (32, 33, 36, 44, 45).

Analyses of *Trps1* expression during mouse tooth organ development, demonstrated that *Trps1* is highly and specifically expressed in the dental mesenchyme (29). After cytodifferentiation, high *Trps1* expression becomes restricted to dental follicle cells, preodontoblasts and odontoblasts. The onset of dentin mineralization coincides with transient downregulation of *Trps1* expression in odontoblasts (29, 46). This dynamic expression pattern suggests that *Trps1* is involved in odontoblast differentiation and function, hence in dentin formation, suggesting that the susceptibility to dental caries reported in TRPS patients might result from formation of compromised dental mineralized tissues. This is further supported by our previous *in vitro* studies of 17IIA11 odontoblast cells showing that *Trps1* deficiency results in loss of their ability to initiate mineralization (47). This was accompanied by significant downregulation of the key osteogenic transcription factor *Osx/Sp7* and other mineralization-related genes in *Trps1*-deficient 17IIA11 cells in comparison with control cells. These *in vitro* studies suggested that *Trps1* is required for odontoblast-driven mineralization.

Two different genetic mouse models were independently generated to study *Trps1* functions in development and mechanisms underlying TRPS pathologies. The first one (*Trps1*<sup>Δgt</sup> mice), harbors an allele with the in-frame deletion of the exon coding for the GATA-type DNA binding domain of *Trps1* (48). The second one harbors a true null allele generated by deleting the first coding exon of *Trps1* (49). *Trps1*<sup>-/-</sup> and *Trps1*<sup>Δgt/Δgt</sup> mice have similar, although not identical, phenotypes. Both models demonstrate delayed cartilage development and endochondral bone formation (34, 48–50), craniofacial skeleton abnormalities (48, 49, 51), and reduced number of hair follicles (48, 49). No apparent tooth developmental abnormalities were observed in *Trps1*<sup>Δgt/Δgt</sup> newborn mice (29), while mild decrease of mineralization in dental tissues was detected by micro-computed tomography (μCT) in 4wk old *Trps1*<sup>+ / Δgt</sup> mice (30). However, due to neonatal lethality in the homozygous form and very mild phenotypes of heterozygous mice, these models are not suitable for studies addressing consequences of *Trps1* deficiency on the postnatal development. This particularly hampers studies of the

tooth, since in mice, development of dental tissues and their mineralization occurs mostly postnatally (52).

This project builds upon the clinical findings of high prevalence of dental caries in TRPS patients, as well as data from *in vitro* studies of *Trps1*-deficient odontoblast cell line and animal models, which collectively suggest an important role of *Trps1* in formation of dentin. We hypothesize that deficiency of *Trps1* in odontoblasts results in impaired quality of dentin and causes a secondary effect on enamel, which makes dental tissues more prone to acid-caused destruction. To investigate the role of the *Trps1* transcription factor in the quality of dental mineralized tissues, we generated a cKO mouse with targeted deletion of the *Trps1* gene in odontoblasts. This new animal model overcomes the limitations of the conventional *Trps1* KO, which do not survive after birth, allowing to study the role of *Trps1* in odontoblasts and postnatal development of dentin.

## MATERIALS AND METHODS

### Animals

Male and female WT and *2.3kbCol1a1-Cre<sup>ERT2</sup>;Trps1<sup>fl/fl</sup>* conditional knockout (*Trps1<sup>Col1a1</sup>cKO*) mice were used. To generate *Trps1* cKO mice, *Trps1* cKO (*Trps1<sup>fl</sup>*) allele was generated by inserting two LoxP sites flanking the first coding exon of *Trps1* by homologous recombination (Figure 1A). The deletion of this exon was used by Suemoto et al. (49), as a strategy to successfully generate the *Trps1* null allele. The *Trps1* cKO construct contained a neomycin resistance cassette (Neo<sup>r</sup>) flanked by FRT sites for a positive selection of recombinant embryonic stem cells (ES). Following the *Trps1* cKO construct injection into C57BL/6 ES cells, neomycin selection was performed, and the resistant ES clones were screened to verify the recombination. Mice carrying the recombinant allele were subsequently obtained *via* the generation of germline chimeras. The *Trps1<sup>fl</sup>* allele was generated after breeding with germline deleter F1p mice (The Jackson Laboratory, strain # 003946)(53), which removed the Neo<sup>r</sup> cassette. *Trps1<sup>Col1a1</sup>cKO* mice were generated by breeding *Trps1<sup>fl</sup>* mice with *2.3kbCol1a1-Cre<sup>ERT2</sup>* mice [B6.Cg-Tg (Col1a1-Cre/ERT2)1Crm/J, The Jackson Laboratory, strain # 016241] expressing Cre recombinase under the control of the 2.3-kb fragment of *Col1a1* promoter (Supplementary Figure 1) (54). The ERT2 domain renders Cre inactive in the cytoplasm, and tamoxifen releases Cre from the ERT2 inhibition allowing its translocation to the nucleus and recombination activity (55, 56). To activate Cre recombinase, tamoxifen (Sigma-Aldrich, # T5648) was administered to all experimental *2.3kbCol1a1-Cre<sup>ERT2</sup>;Trps1<sup>fl/fl</sup>* and control WT (*Trps1<sup>fl/fl</sup>*) mice *via* intraperitoneal injection (0.1 mg/g body weight) at postnatal days (P)1, P2, P9, P16 and P23 to assure efficient deletion of *Trps1* in odontoblasts and as a control in WT mice (Figure 1C). Tamoxifen was dissolved in corn oil to a concentration of 10 mg/ml. mTmG [B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J, The Jackson Laboratory, strain # 007676] crossed with *2.3kbCol1a1-Cre<sup>ERT2</sup>* mice were used to verify Cre-mediated recombination by microscopic imaging. Genotyping was carried out on the DNA extracted from tail biopsies using PCR with the following

primers: WT and *Trps1<sup>fl</sup>* allele (F: CCCATAGCACTTATTTA GTCCAG; R: CCTATCCTTTGTAACCTAACTCTC), *Coll1a1-Cre* (F: CTCAGAGCTGTTATTTATTA; R: CATCGACCGG TAATGCAG) (57) (Figure 1B). The mice were kept on a caries-susceptible C57BL/6J genetic background (58).

All animal studies were conducted in accordance with a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC protocol # 19095648), complying with the Federal Animal Welfare Act and all NIH policies regarding vertebrate animals in research. Mice were euthanized by CO<sub>2</sub> inhalation. All analyses were performed on tissues collected post-mortem.

## Histology

Hemimandibles of 4 wk old WT and *Trps1<sup>Coll1a1</sup>cKO* male and female mice ( $N = 3/\text{genotype/sex}$ ) were dissected under a stereo microscope (Leica S9D) to remove soft tissues, and fixed with 10% formalin (Fisher, # S7100-4) overnight. Samples were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) solution (pH 7.4) for 14 days prior to paraffin embedding. Serial 7  $\mu\text{m}$  sagittal sections were placed on Fisherbrand™ Superfrost™ Plus microscope slides and deparaffinized for hematoxylin and eosin (H&E) staining following standard protocols. Hemimandibles of P7 and 4 wk old *2.3kbColl1a1-Cre<sup>ERT2</sup>;mTmG* reporter mice were harvested and processed as described above. Decalcified samples were cryoprotected in 30% sucrose/PBS overnight at 4 °C, embedded in an OCT compound and stored at -80 °C until sectioned. Samples were cryosectioned at 7  $\mu\text{m}$ , protected from the light. To validate Cre recombinase activation in odontoblasts of mandibular first molars of reporter mice, cryosections were counterstained with DAPI and mounted with immu-mount (Fisher, # 9990402) for microscopic analyses of GFP and RFP signals.

## Microscopy

Whole teeth images were captured on a Leica M165FC dissecting microscope using a DFC 450 camera and Leica LAS software. Histological images were captured on a Zeiss AXIO microscope with an AxioCam MRc 35 camera and Zen software. Microindentation images were captured using a BUEHLER® IndentaMet™1100 Series microindentation hardness tester adapted to a uEye camera and a Buehler Omnimet MHT software.

## $\mu\text{CT}$ Scanning and Analyses

For the densitometric and volumetric analysis of mineralized tissues of mandibular first molars, hemimandibles of 4wk old mice ( $N = 5/\text{genotype/sex}$ ) were imaged in 70% ethanol by the Scanco  $\mu\text{CT}$  50 (Scanco Medical, Brüttisellen, Switzerland) system. The following parameters were set for the scans: 6- $\mu\text{m}$  voxel size, 55 KVp, 0.36 degrees rotation step (180 degrees angular range) and a 1,500 ms exposure per view. After 3D reconstruction, volumes were segmented using a global threshold of 0.6 g HA/cc. Mineral density (TMD), thickness (Th), and volumes (BV) were measured for enamel and dentin separately. Additionally, dentin and enamel tissue fraction (BV/TV) in the

total tooth crown volume (TV) was calculated as described before (59).

## Dental Tissues Microhardness Tests

Each first molar was extracted from the hemimandible after  $\mu\text{CT}$  scanning, mounted in Epofix (EMS, Hatfield, PA) and polished as previously described (60). Mechanical hardness was measured by doing three indentations with a Vickers diamond (25 g load during 5 s dwell time) using a microhardness tester (IndentaMet 1,100 Series, Buehler Ltd., Lake Bluff, IL, USA), on polished enamel (inner and outer) and dentin (mantle and circumpulpal) in the occlusal, middle, and cervical third of the crown. All indentations were made perpendicularly to the external surface of each specimen (61). Additionally, indentations in the enamel and dentin localized in the pit were made at a distance of at least three indentations diagonal from each other. The resulting indentations were measured under a microscope. Vickers hardness values were calculated using the following formula:  $HV = 1.854 \times F/d^2$ . With F being the applied load (measured in kg-force) and  $d^2$  the area of the indentation (measured in  $\text{mm}^2$ ) (62). Microhardness of each specific region of the enamel and dentin was expressed as a mean value of  $N=3$  indentations.

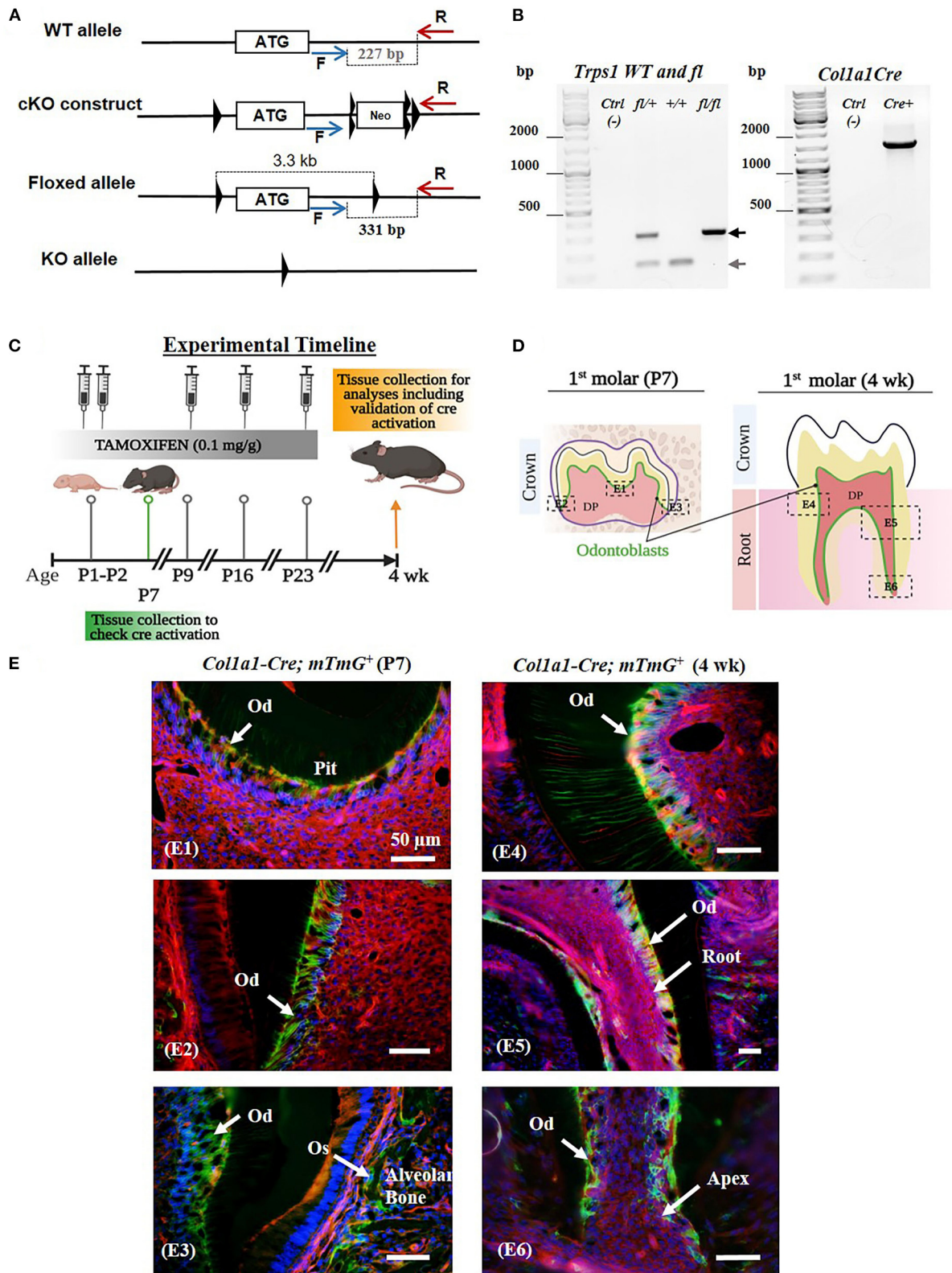
## Artificial Caries Procedure

Molars of 4wk old WT and *Trps1<sup>Coll1a1</sup>cKO* mice ( $N=5/\text{genotype/sex}$ ) were analyzed using the protocol described by Vieira et al., (61). This method produces a subsurface enamel lesion instead of surface erosion (61). Briefly, following baseline microhardness measurements of enamel and crown dentin, artificial caries lesions were created by immersing each mounted molar in demineralizing solution (1.3 mmol/L Ca, 0.03  $\mu\text{g}$  F/mL, 0.05 mol/L acetate buffer, phosphoric acid to adjust pH 5.0) at 37°C for 16 h. The post-demineralization indentations were created right underneath the baseline indentations on proximal surfaces, or next to the initial one in molar pits at a distance of at least one indentation diagonal from each other. We analyzed surface microhardness of inner and outer enamel, mantle and circumpulpal dentin, in addition to enamel and crown dentin in pits. The susceptibility to dental caries was expressed as a difference between mean values of pre- and post-demineralization mechanical hardness.

## Statistical Analyses

Experiments performed in this study used five mice per genotype per sex or otherwise stated. Males and females were analyzed separately. Values are expressed as mean  $\pm$  standard deviation (SD). Statistically significant differences were determined using the Student's *t*-test. A *p*-value of  $< 0.05$  was considered statistically significant. Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA).





**FIGURE 1 |** Generation of *Trps1<sup>Col1a1</sup>* cKO mice and verification of Cre-mediated recombination in odontoblasts. **(A)** The conditional knockout strategy: schematics of *Trps1* WT allele, targeting construct, and floxed allele showing the first coding exon (the white box with the ATG) and relative localization of LoxP sites (triangles), FRT (Continued)

**FIGURE 1** | sites (double triangles) and primers for genotyping PCRs (blue and red arrows). **(B)** Examples of genotyping PCR results: Floxed (*fl*; 331 bp) and WT (227 bp) alleles were detected by PCR with F and R primers, *Col1a1-Cre* transgene was detected with primers encompassing *Col1a1* promoter fragment and Cre sequence (57). **(C)** Experimental timeline showing the age of mice during tamoxifen administration, timing and dose of tamoxifen injections, and tissue collections. **(D)** Schematics of P7 and 4 wk old mouse molars showing the areas analyzed in panel E (dotted box); localization of cells of interest (odontoblasts) is shown in green. **(E)** Validation of the experimental timeline of tamoxifen injections and Cre-mediated recombination in odontoblasts using ROSA<sup>mTmG</sup> reporter mice. Fluorescent microscopy images showing efficient and specific tamoxifen-induced Cre-mediated recombination (GFP+ signal; green and yellow cells) in odontoblasts (Od) and osteoblasts (Os) of mandibular first molars and alveolar bone, respectively. Red fluorescent cells are recombination negative. DAPI was used to stain nuclei (blue). (E1) Occlusal pit of P7 mouse molar, (E2) P7 crown dentin, (E3) P7 crown dentin and alveolar bone, (E4) 4 wk crown dentin, (E5) 4 wk molar root, (E6) 4 wk molar root apex. Scale bar = 50 μm.

## RESULTS

### Generation of 2.3kb*Col1a1-Cre*<sup>ERT2</sup>;*Trps1*<sup>fl/fl</sup> Conditional Knockout (*Trps1*<sup>Col1a1</sup> cKO) Mice

Neonatal lethality of the current mouse models of *Trps1* deficiency (48, 49), limits *in vivo* studies of *Trps1* function to embryonic development. To enable the research addressing the role of *Trps1* in postnatal development and in specific cell types, we generated *Trps1* cKO mice, in which the first coding exon of *Trps1* is flanked by LoxP sites (*Trps1*<sup>fl</sup> mice; **Figure 1A**). To determine consequences of *Trps1* deficiency on quality of dental tissues, which are formed postnatally in mice, and gain insights into the mechanism underlying increased susceptibility to dental caries in TRPS, we generated mice with deficiency of *Trps1* in odontoblasts (*Trps1*<sup>Col1a1</sup> cKO mice). *Trps1* knockout was initiated with tamoxifen injections at postnatal day 1 (P1) and P2, followed by 3 more injections 7 days apart (**Figure 1C**) (63–65). To verify the efficiency of this tamoxifen administration scheme in activating Cre recombination in odontoblasts, we used 2.3kb*Col1a1-Cre*<sup>ERT2</sup>;*mTmG* reporter mice. Cre recombination was evaluated in molars of P7 and 4wk old reporter mice (**Figures 1D,E**). Fluorescent microscopy analyses detected cell membrane-localized GFP+ (green and yellow) signals in the odontoblast layer and RFP+ (red) signals in the remaining pulp cells. GFP+ signals were also detected in osteoblasts lining the alveolar bone, which is another cell type expressing Cre from the 2.3kb*Col1a1* promoter (54). These results demonstrate that our tamoxifen administration scheme efficiently induced Cre recombination in odontoblasts as well as in osteoblasts in the alveolar bone (**Figures 1D,E**).

### Gross Characterization of *Trps1*<sup>Col1a1</sup> cKO Mice

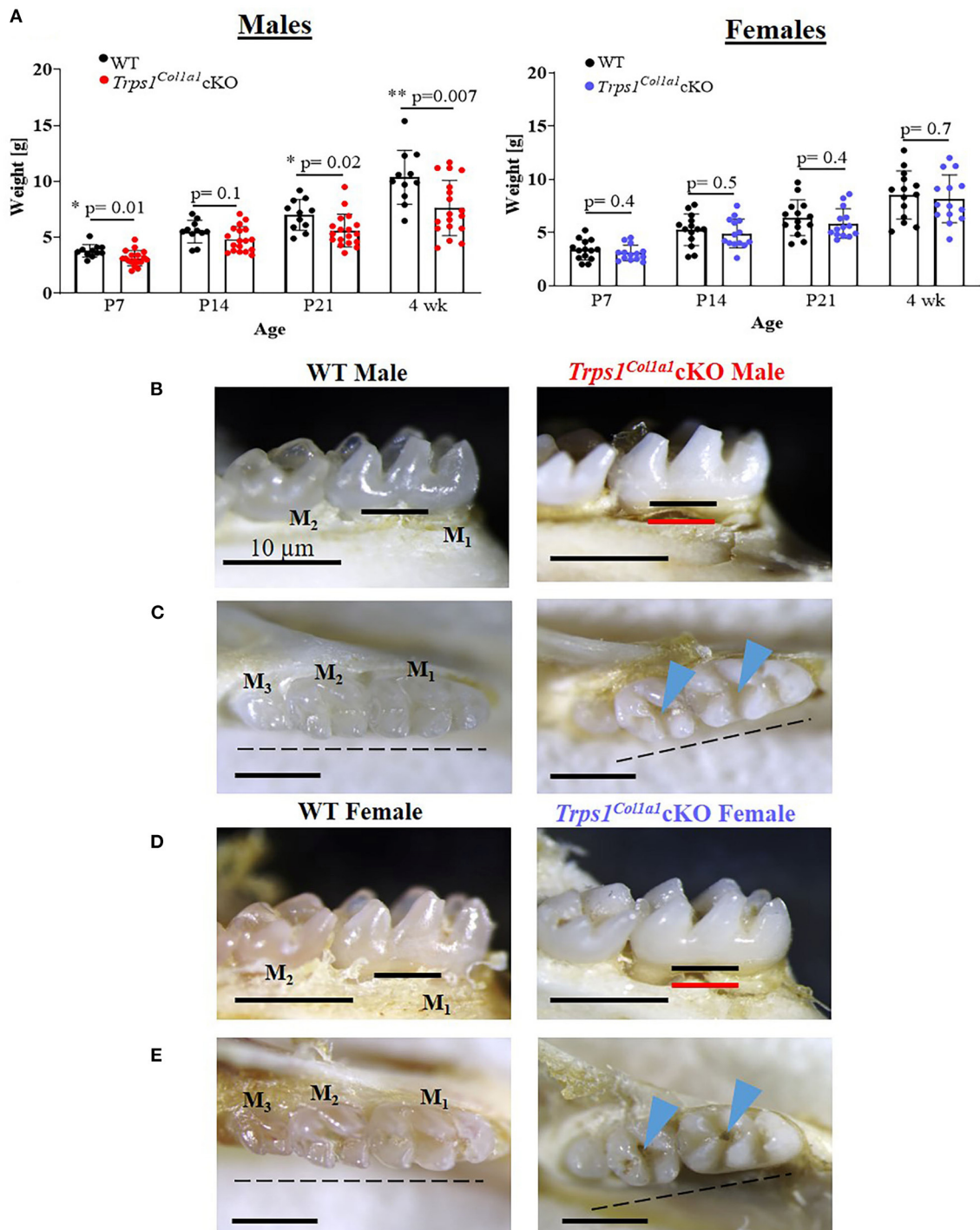
Body weight measurements of WT and *Trps1*<sup>Col1a1</sup> cKO mice demonstrated that *Trps1*<sup>Col1a1</sup> cKO males are significantly smaller than WT littermates at P7, P21 and 4 wks of age. This difference was not found in females, suggesting that *Trps1* deficiency has stronger effect in males than in females (**Figure 2A**). Gross examination detected no aberrations in the tooth number in *Trps1*<sup>Col1a1</sup> cKO and no apparent differences in the tooth crown morphology compared to WT mice. However, analysis of the dental phenotype under a dissecting microscope showed that teeth of 4 wk old mice were “chalky” white and opaque in contrast to the translucent aspect observed in molars of WT mice, irrespective of the sex (**Figures 2B–E**). Such

“chalky” appearance of teeth suggests hypomineralization of dental tissues (66, 67). Additionally, both *Trps1*<sup>Col1a1</sup> cKO male and female mice presented severe root exposure at 4 wks of age, suggesting impaired formation of the alveolar bone (**Figures 2B,D**). Misalignment of the molars was also detected in *Trps1*<sup>Col1a1</sup> cKO males and females. Furthermore, more detailed microscopic examination of the occlusal surface of mandibular molars revealed deep pits in *Trps1*<sup>Col1a1</sup> cKO mice (**Figures 2C,E**; blue arrowheads), which suggests defects in crown mineralized tissues formation.

### *Trps1* Deficiency in Odontoblasts Impairs Formation of Dental Mineralized Tissues

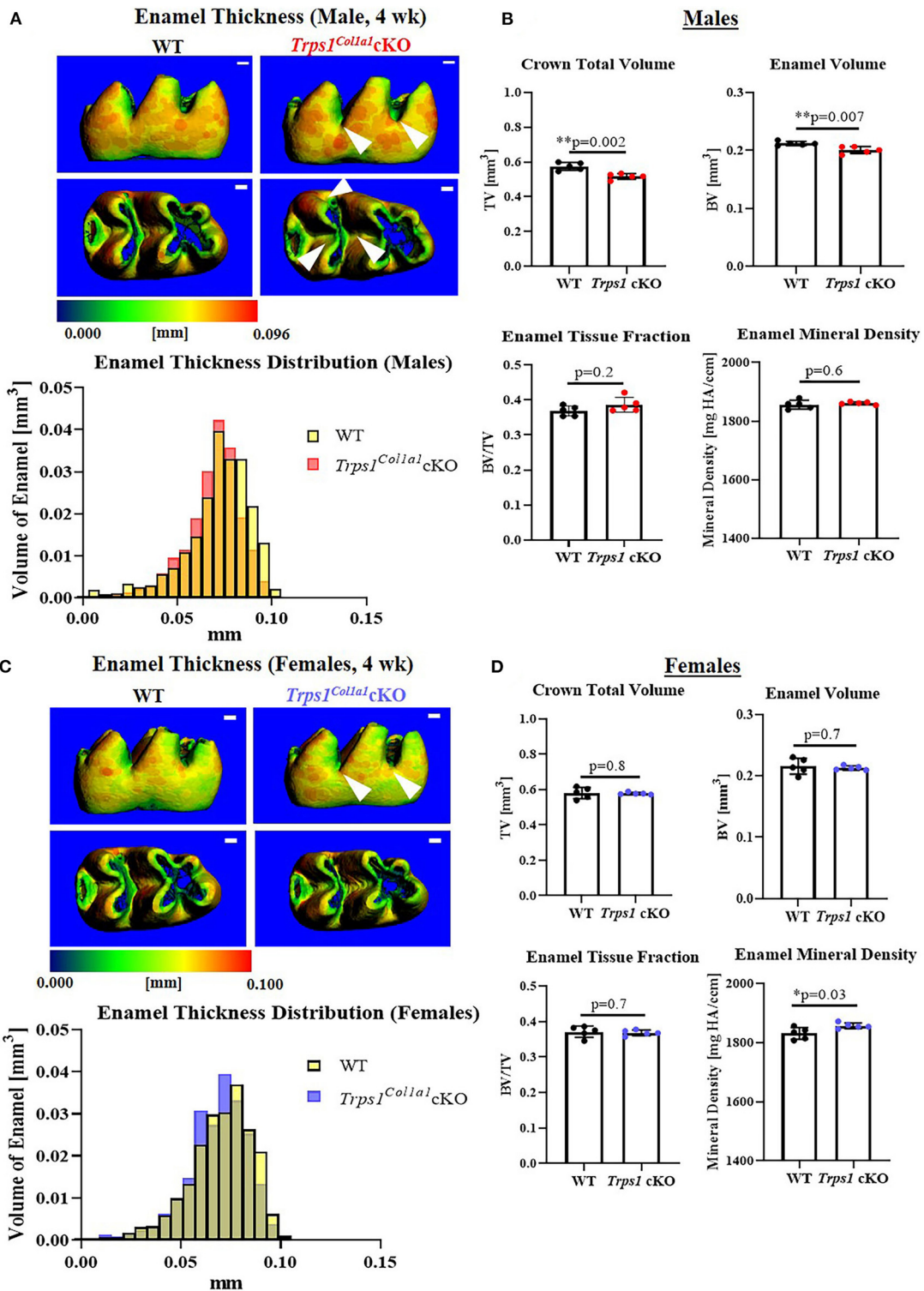
To understand the effect of the odontoblast-specific deficiency of *Trps1* on the quality of the tooth crown, we performed quantitative μCT analyses of enamel and crown dentin volume, tissue thickness, tissue fraction in the total crown volume, and mineral density in mandibular first molars of WT and *Trps1*<sup>Col1a1</sup> cKO 4wk old mice. Results of μCT analyses of enamel thickness on smooth surfaces of molars showed no overall difference between WT and *Trps1*<sup>Col1a1</sup> cKO mice (**Figures 3A,C**, top panel images). However, pseudo-coloring of 3D μCT images, based on the tissue thickness in each area, revealed pronounced buccal pits in male and female *Trps1*<sup>Col1a1</sup> cKO molars (**Figures 3A,C**, white arrowheads). Furthermore, reduced enamel thickness in occlusal pits became evident in *Trps1*<sup>Col1a1</sup> cKO males (**Figure 3A**, bottom panel image; **Supplementary Figure 2**). Interestingly, these areas correspond to locations particularly susceptible to dental caries in humans. The crown total volume and enamel volume were significantly reduced only in *Trps1*<sup>Col1a1</sup> cKO males (**Figure 3B**), suggesting that their teeth are smaller compared to WT. There was no difference in enamel mineral density in males (**Figure 3B**), but, surprisingly, it was significantly higher in *Trps1*<sup>Col1a1</sup> cKO females compared to WT (**Figure 3D**). Since in *Trps1*<sup>Col1a1</sup> cKO mice, *Trps1* is deleted in odontoblasts, and ameloblasts do not express *Trps1* (29), these results suggest that enamel defects in *Trps1*<sup>Col1a1</sup> cKO mice result from a cell non-autonomous mechanism.

μCT analyses of crown dentin showed significantly reduced dentin volume and tissue fraction in male and female *Trps1*<sup>Col1a1</sup> cKO molars in comparison to WT (**Figures 4, 5**). Decreased dentin quantity was further demonstrated by pseudo-coloring of 3D μCT images, which showed reduced dentin thickness throughout the crown in *Trps1*<sup>Col1a1</sup> cKO males and females (**Figures 4C, 5C**; **Supplementary Figure 3**). μCT



**FIGURE 2 |** Gross assessment of *Trps1<sup>Col1a1</sup>cKO* mice and appearance of teeth. **(A)** Body weight of WT and *Trps1<sup>Col1a1</sup>cKO* mice (males,  $N = 11$  and  $N = 18$ , respectively; females,  $N = 14$  and  $N = 14$ , respectively) showing mild growth retardation of *Trps1<sup>Col1a1</sup>cKO* males. Data are shown as individual weight of each mouse (represented by dots) along with mean values  $\pm$  SD; \* $p < 0.05$  and \*\* $p < 0.01$ . **(B, D)** Lingual view of mandibular molars (M<sub>1</sub> and M<sub>2</sub>) showing “chalky” white appearance and exposed root surfaces in 4 wk old *Trps1<sup>Col1a1</sup>cKO* male and female mice. The black line represents the bone level at baseline, and the red line represents the bone level in the *Trps1<sup>Col1a1</sup>cKO*. **(C, E)** Occlusal view of mandibular molars (M<sub>1</sub>, M<sub>2</sub>, M<sub>3</sub>) showing deep molar pits (blue arrowheads) and misaligned teeth (dotted line) in 4 wk old *Trps1<sup>Col1a1</sup>cKO* males and females. Scale bar = 10  $\mu$ m.





**FIGURE 3 |** Quantitative  $\mu$ CT analyses of the enamel of mandibular first molars of 4 wk old WT and *Trps1<sup>Col1a1</sup> cKO* mice. **(A,C)** Representative 3D  $\mu$ CT images of the enamel; scale bar = 100  $\mu$ m. The enamel was pseudo-colored based on the distribution of tissue thickness. The corresponding tissue thickness color scale is shown below the images. White arrowheads point at the reduced enamel thickness specifically in pits of *Trps1<sup>Col1a1</sup> cKO* male molars. There was no noticeable difference between WT and *Trps1<sup>Col1a1</sup> cKO* females. The graph below the 3D images shows the comparison of the quantity (volume) of the enamel with specific thickness in WT and *Trps1<sup>Col1a1</sup> cKO*. **(B,D)** Quantification of enamel volume, enamel tissue fraction in the total crown volume, and mineral density. Mean values  $\pm$  SD and individual data points (dots, N=5/genotype/sex) are shown; \* $p < 0.05$  and \*\* $p < 0.01$ .



analyses did not detect significant differences in crown dentin mineral density (Figures 4B, 5B). However, histological analyses of molars uncovered wider predentin, the presence of globular dentin (Figures 6A,B) and pronounced occlusal pits, especially in *Trps1<sup>Coll1a1</sup>* cKO males (Figures 2C,E, 6C). Furthermore,  $\mu$ CT 2D images (Figures 4A, 5A) revealed enlarged pulp chambers and prominent pulp horns in both *Trps1<sup>Coll1a1</sup>* cKO male and female mice, while  $\mu$ CT 3D images demonstrated some cases of attrition (Supplementary Figure 4) in *Trps1<sup>Coll1a1</sup>* cKO mice. In summary, the  $\mu$ CT and histological analyses of molars from 4wk old mice suggest that deficiency of *Trps1* in odontoblasts impairs dentin formation, which, in turn, compromises the enamel.

## Odontoblast-Specific *Trps1* Deficiency Reduces Dentin and Enamel Microhardness and Increases Mineral Loss in Acidic Conditions

To determine whether the deficiency of *Trps1* in odontoblasts affects the quality of dental tissues, we analyzed enamel and crown dentin hardness in 4 wk old WT and *Trps1<sup>Coll1a1</sup>* cKO male and female mice using the Vickers microhardness test. We analyzed separately outer and inner enamel, and mantle and circumpulpal dentin. The images of the indentations performed with a Vickers diamond in the enamel and dentin illustrate differences between WT and *Trps1<sup>Coll1a1</sup>* cKO tissues (Figures 7A, 8A, 9A,B). Results of Vickers microhardness measurements (HV values) demonstrated that odontoblast-specific *Trps1* deficiency resulted in formation of softer (lower HV values) enamel and dentin (Figures 7, 8; Table 1). Specifically, the outer enamel and circumpulpal dentin were significantly softer at the baseline in *Trps1<sup>Coll1a1</sup>* cKO males and females than in WT mice (Figures 7B, 8B; Table 1).

Since dental caries is caused by the release of organic acids from fermentative bacteria, which results in the dissolution of hydroxyapatite from enamel and dentin (4), our next step was to determine whether the quality of *Trps1<sup>Coll1a1</sup>* cKO enamel and dentin affects their susceptibility to acid-induced demineralization. For that, we specifically selected an *ex vivo* artificial caries approach to eliminate multiple variables contributing to development of dental caries and specifically focus on quality of dental tissues. To determine the effectiveness of the artificial caries procedure, we did two different types of analyses. First, we compared HV values at baseline and post-demineralization in each tissue (Table 1). Second, we calculated the percentage of microhardness loss during the artificial caries procedure in each analyzed tissue (Figures 7C, 8C, 9E,F; Table 1).

After acid-induced demineralization, in WT mice, only dentin microhardness was significantly lower compared to baseline, while in *Trps1<sup>Coll1a1</sup>* cKO mice both dentin and enamel were significantly affected (Table 1). This demonstrated that the *ex vivo* demineralization procedure was effective. More importantly, these analyses revealed that *Trps1<sup>Coll1a1</sup>* cKO tissues were more prone to acid-induced demineralization than WT tissues. This is further highlighted by the calculations of the percentage of microhardness loss, which revealed stronger demineralization

(loss of hardness) specifically in the circumpulpal dentin, pit dentin and outer enamel in *Trps1<sup>Coll1a1</sup>* cKO males (Figures 7C, 9E; Table 1); as well as in the inner enamel in *Trps1<sup>Coll1a1</sup>* cKO females in comparison with WT mice (Figure 8C).

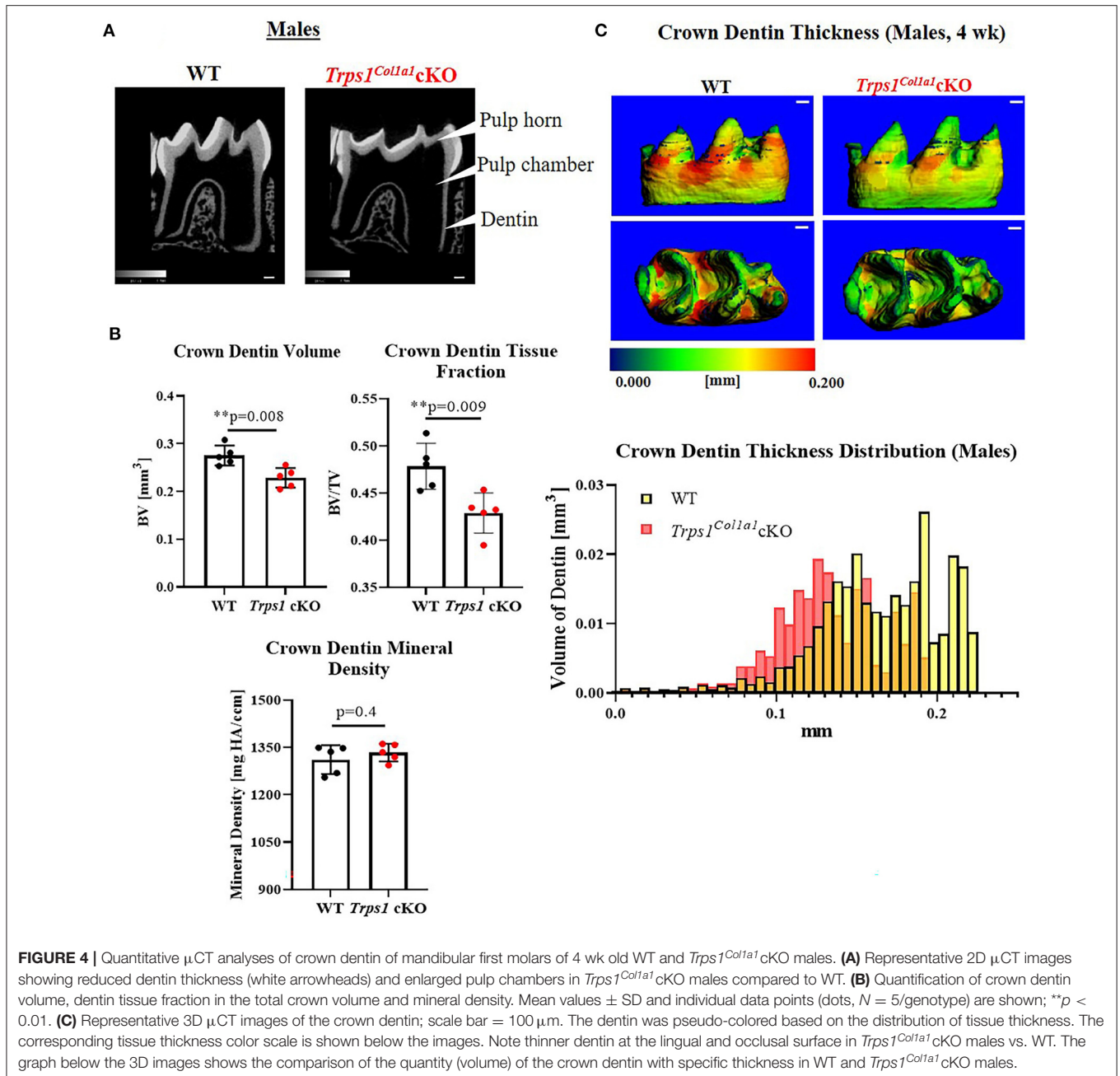
In the molar pits (Figure 9), enamel of *Trps1<sup>Coll1a1</sup>* cKO males had significantly lower HV values than in WT males and *Trps1<sup>Coll1a1</sup>* cKO females at baseline and post-acid demineralization (Figures 9C,D; Table 1), demonstrating overall softer tissues in the pits of *Trps1<sup>Coll1a1</sup>* cKO male molars.

In summary, results of the microhardness analyses at baseline and after acid-induced demineralization demonstrate that odontoblast-specific *Trps1* deficiency not only decreases enamel and crown dentin microhardness, but also results in formation of dental tissues that are less resistant to demineralization in acidic conditions.

## DISCUSSION

The goal of this study was to provide insights into the pathology underlying the increased susceptibility to dental caries phenotype in TRPS patients (36, 44). Considering that: (i) *Trps1* is expressed in odontoblasts (29, 30, 46), (ii) *Trps1* haploinsufficiency impairs tooth mineralization in mice (30), and (iii) deficiency in odontoblast cell line reduces expression of multiple mineralization-related genes (47), we focused on the role of *Trps1* in the odontoblast function—formation of dentin, and the consequences of *Trps1* deficiency on the quality of the tooth crown tissues. For that, we generated a new animal model—*Trps1<sup>Coll1a1</sup>* cKO mice, in which *Trps1* is deleted in odontoblasts. Our  $\mu$ CT analyses of mouse molars revealed that odontoblast-specific deletion of *Trps1* leads to decreased crown dentin volume, while no significant differences were detected in dentin mineral density. However, histological analyses revealed a wider predentin and globular dentin pattern in *Trps1<sup>Coll1a1</sup>* cKO mice, which are indicative of disturbed dentin mineralization (68). Interestingly, the functional *ex vivo* assays assessing dental tissues hardness and their susceptibility to acid-induced demineralization revealed that not only dentin, but also enamel hardness was decreased in *Trps1<sup>Coll1a1</sup>* cKO mice, with some sex-specific differences. Together, the analyses of *Trps1<sup>Coll1a1</sup>* cKO mice suggest that compromised quality of dental tissues as well as decreased dentin thickness, particularly in the tooth crown regions susceptible to caries, contribute to a high prevalence of dental caries in TRPS patients.

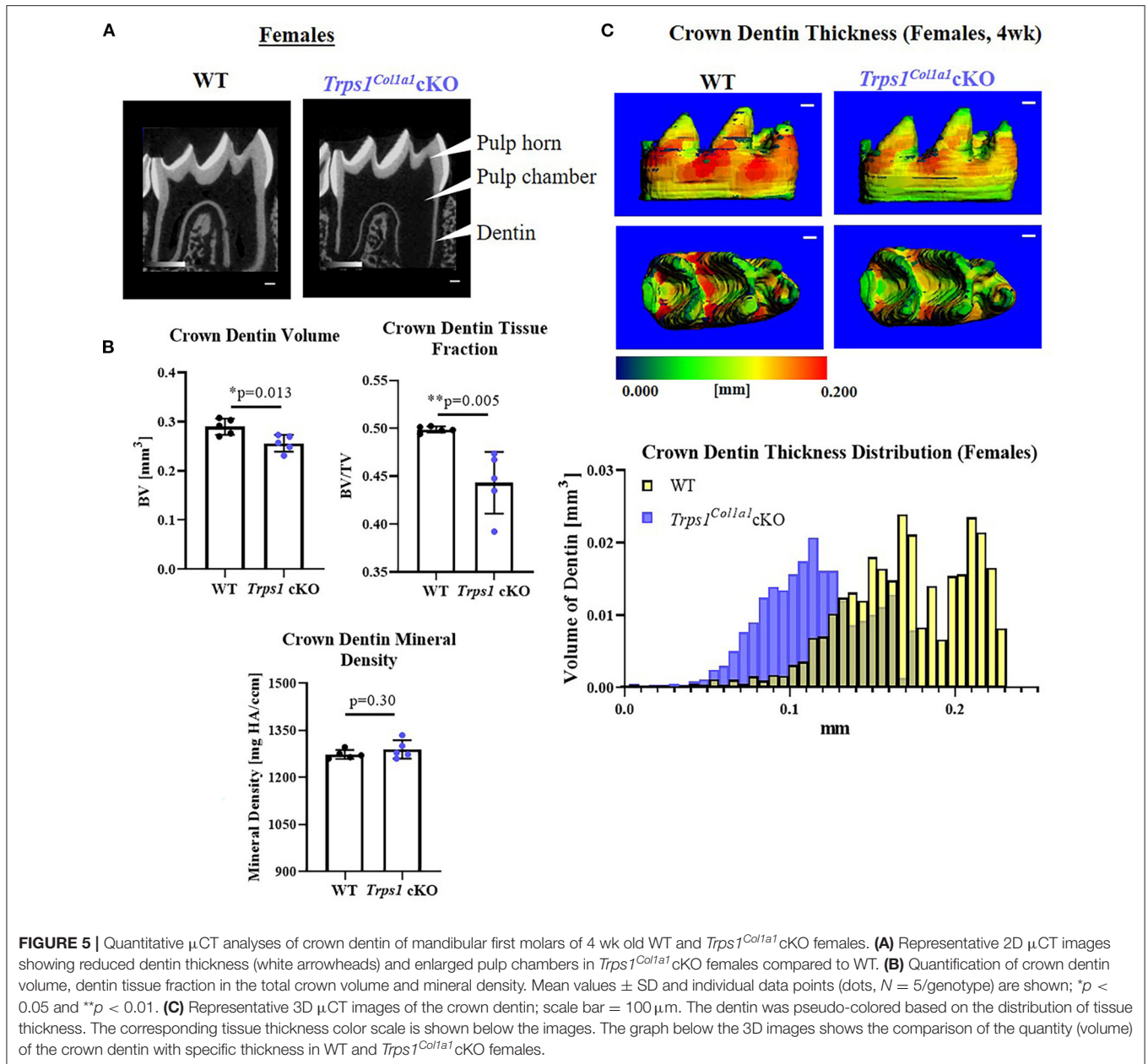
Whether or not an individual experiences dental caries depends on a large range of endogenous factors (e.g., enamel quality, tooth morphology, and saliva composition and flow rate); behavioral features (e.g., diet and oral hygiene); socioeconomic and demographic factors (e.g., age, sex, race); environmental exposures (e.g., oral bacteria and fluoride); along with host-genetics (11, 13, 18, 69). Candidate genes associated with dental caries reported so far include: immune response genes, genes related to taste, those related to saliva flow rates and composition, as well as genes involved in tooth formation and mineralization (17). Using the cKO approach with *Trps1* deletion only in developed odontoblasts, we focused on



investigating the role of *Trps1* transcription factor as potential contributor to susceptibility to dental caries. Our analyses revealed several characteristics of *Trps1<sup>Coll1a1</sup>cKO* teeth that may render them more prone to the initiation and progression of dental caries.

First, quantitative  $\mu$ CT analyses demonstrated that dentin in *Trps1<sup>Coll1a1</sup>cKO* molars is thinner than in WT mice. This deficiency was particularly prominent in pits of the tooth crown, which are the sites most susceptible to dental caries initiation in humans. Teeth with thinner dentin may be prone to more severe caries, as once a lesion is initiated, it can reach the pulp faster than in teeth with a thicker dentin layer. On radiographic images

of teeth, features such as enlarged pulp chambers and prominent pulp horns suggest a thinner dentin layer. This was visible on 2D  $\mu$ CT images of *Trps1<sup>Coll1a1</sup>cKO* molars, and importantly, such features were recently reported in permanent molars of a 16-year-old male patient with *TRPS1* mutation (32). The authors of this report proposed that large pulp chambers in this individual are a consequence of the *TRPS1* mutation. The detection of the same characteristics in *Trps1<sup>Coll1a1</sup>cKO* molars supports this conclusion and ratifies *Trps1<sup>Coll1a1</sup>cKO* mice as a suitable animal model for studying *Trps1* function in odontoblasts and molecular pathologies underlying some of the dental phenotypes in TRPS.

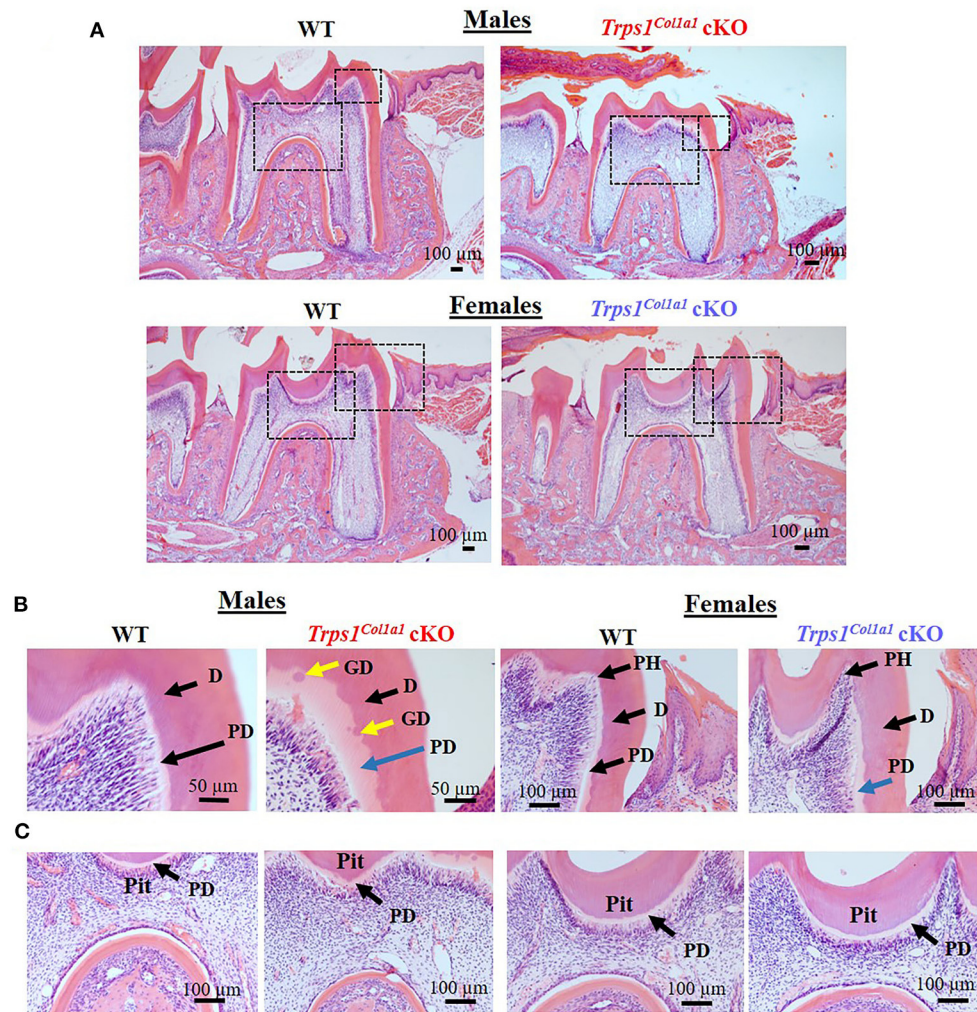


**FIGURE 5 |** Quantitative  $\mu$ CT analyses of crown dentin of mandibular first molars of 4 wk old WT and *Trps1*<sup>Coll1a1</sup>cKO females. **(A)** Representative 2D  $\mu$ CT images showing reduced dentin thickness (white arrowheads) and enlarged pulp chambers in *Trps1*<sup>Coll1a1</sup>cKO females compared to WT. **(B)** Quantification of crown dentin volume, dentin tissue fraction in the total crown volume and mineral density. Mean values  $\pm$  SD and individual data points (dots,  $N = 5$ /genotype) are shown; \* $p < 0.05$  and \*\* $p < 0.01$ . **(C)** Representative 3D  $\mu$ CT images of the crown dentin; scale bar = 100  $\mu$ m. The dentin was pseudo-colored based on the distribution of tissue thickness. The corresponding tissue thickness color scale is shown below the images. The graph below the 3D images shows the comparison of the quantity (volume) of the crown dentin with specific thickness in WT and *Trps1*<sup>Coll1a1</sup>cKO females.

Second, tissue microhardness and *ex vivo* artificial caries experiments uncovered that both dentin as well as enamel of *Trps1*<sup>Coll1a1</sup>cKO teeth are more prone to acid-induced demineralization than WT tissues. This suggests that in TRPS patients, acid produced by oral bacterial fermentation of dietary carbohydrates can destroy dental tissues easier than in healthy individuals, hence making them more prone to the initiation of dental caries as well as enables caries progression. Formation of teeth which are softer and more susceptible to acid demineralization in *Trps1*<sup>Coll1a1</sup>cKO mice, along with thinner dentin, strongly suggests that compromised quality of dental tissues contributes to increased incidence and severity of dental caries in TRPS patients.

Interestingly, and highly relevant to the development of dental caries, the enamel quality is also impaired by *Trps1* deficiency in odontoblasts, as evident from lower enamel microhardness, and some cases of attrition in *Trps1*<sup>Coll1a1</sup>cKO mice. The outer enamel (OE) of both *Trps1*<sup>Coll1a1</sup>cKO males and females is significantly softer, than in the WT mice; while the inner enamel (IE) hardness of *Trps1*<sup>Coll1a1</sup>cKO females becomes significantly lower after the artificial caries procedure as compared to WT. Notably, the detection of localized enamel mineralization defects in pits contributes to our understanding of dental caries lesion distribution pattern in TRPS. We agree with others, that the effects of dental caries risk factors may be surface-specific (10). Most likely, in *Trps1*<sup>Coll1a1</sup>cKO mice, the enamel mineral



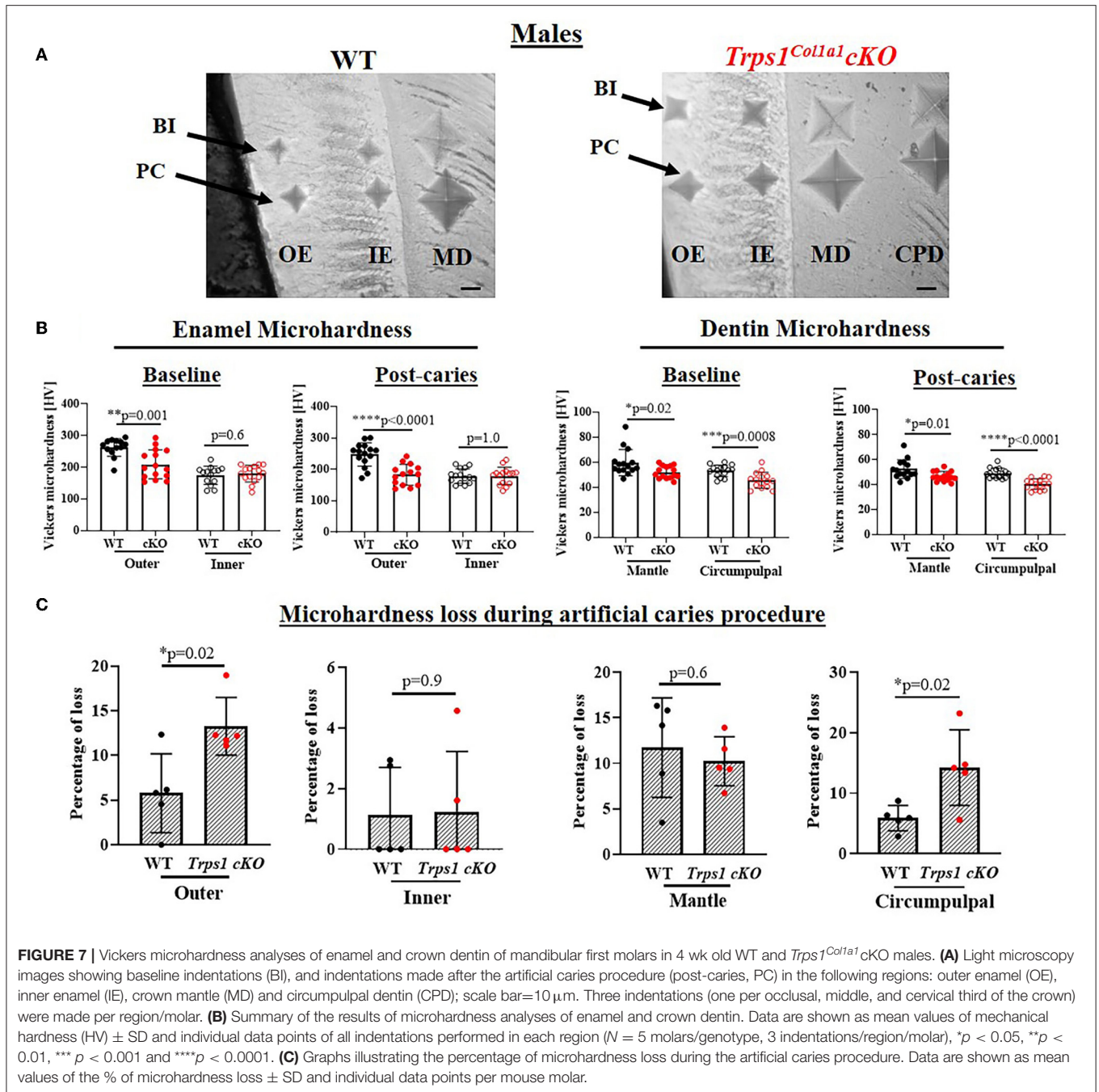


**FIGURE 6 |** Histological evaluation (H&E staining) of the mandibular first molar crown tissues in 4wk old WT and *Trps1<sup>Coll1a1</sup> cKO* mice. **(A)** Low magnification images showing gross histological appearance of molars. Boxed areas are shown in higher magnification on panels B and C. **(B)** High magnification of the mesial cusp showing wider predentin in *Trps1<sup>Coll1a1</sup> cKO* males (blue arrow) and irregular mineralization of crown dentin with calcospherites (globular dentin, yellow arrow). Note the pronounced pulp horns (arrows) in *Trps1<sup>Coll1a1</sup> cKO* females. **(C)** High magnification of the pit area. Note the deeper appearance of the pit in *Trps1<sup>Coll1a1</sup> cKO* males. Pulp horn (PH), dentin (D), predentin (PD), globular dentin (GD).  $N = 3$  mice/genotype were analyzed.

maturation stage, and not the secretory ameloblast functions, are compromised, since no difference in the enamel thickness was detected in most of the surfaces of the first molars. The effect of the odontoblast-specific deletion of *Trps1* on the enamel is not surprising, as there is evidence that formation of enamel is influenced by developing dentin. The importance of proper dentin development for formation of enamel is underscored by severe enamel defects in genetic dentin mineralization disorders such as dentinogenesis imperfecta II, and hypophosphatemic rickets (19, 70–72). Additionally, the differentiation and function of ameloblasts is regulated by signaling from odontoblasts (73). For example, hedgehog and Wnt signaling pathways, which are implicated in molecular communication between developing odontoblasts and ameloblasts, have been shown to be regulated by *Trps1* in developing bones and hair follicles (34, 50, 74–76).

Hence, these signaling pathways may be disturbed in *Trps1*-deficient odontoblasts, affecting ameloblast function and enamel formation. Furthermore, *Trps1* is essential for odontoblast ability to release matrix vesicles (MVs), as shown in our previous *in vitro* studies (47). Although, in mineralized tissues, the MVs main function is to support the initiation of mineralization, there is growing evidence that, like other extracellular vesicles, MVs also participate in the intercellular communication (77). Hence, the formation of weaker enamel in *Trps1<sup>Coll1a1</sup> cKO* mice results from cell non-autonomous mechanisms, secondary to either defective dentin formation or signaling from *Trps1*-deficient odontoblasts.

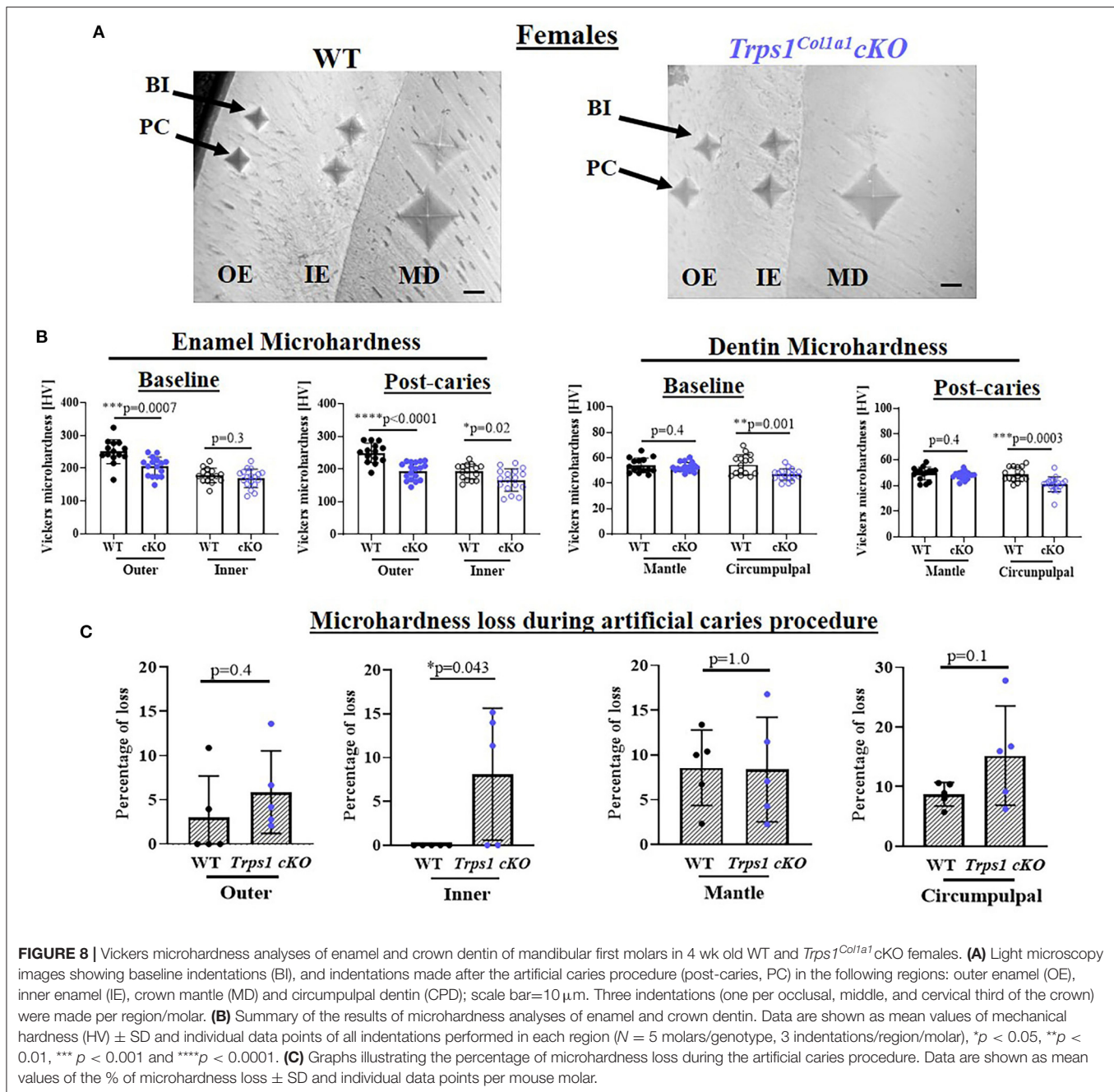
We did not detect significant differences in dentin and enamel mineral density of *Trps1<sup>Coll1a1</sup> cKO* molars versus WT, although correlation between microhardness and mineral content has been reported in human enamel (78). However, the mechanical



hardness of mineralized tissues does not depend solely on the mineral content; it depends also on the organization of the mineral (hydroxyapatite crystals) in the ECM (79). It is likely then, that *Trps1* deficiency affects the organization of hydroxyapatite crystals but not the mineralization extent. This will be addressed in a follow-up study of the composition and structure of dental tissues in *Trps1<sup>Coll1a1</sup> cKO* mice.

Consistent with our previous *in vitro* studies (47), we detected impaired initiation of the dentin mineralization in *Trps1* deficiency. This was detected through histological analyses as

the presence of globular dentin and enlarged predentin, which are apparent on the microscopic images. The presence of the globular dentin in *Trps1<sup>Coll1a1</sup> cKO* mice, along with widened predentin and thin mineralized dentin, resembles mineralization defects observed in hypophosphatemic rickets—a rare disease caused by mutations in phosphate homeostasis genes (80). While such dentin mineralization defects in hypophosphatemic rickets are caused by systemic phosphate deficiency (81, 82), our previous studies of *Trps1* function in odontoblasts indicate that similar phenotype can be caused by cell autonomous

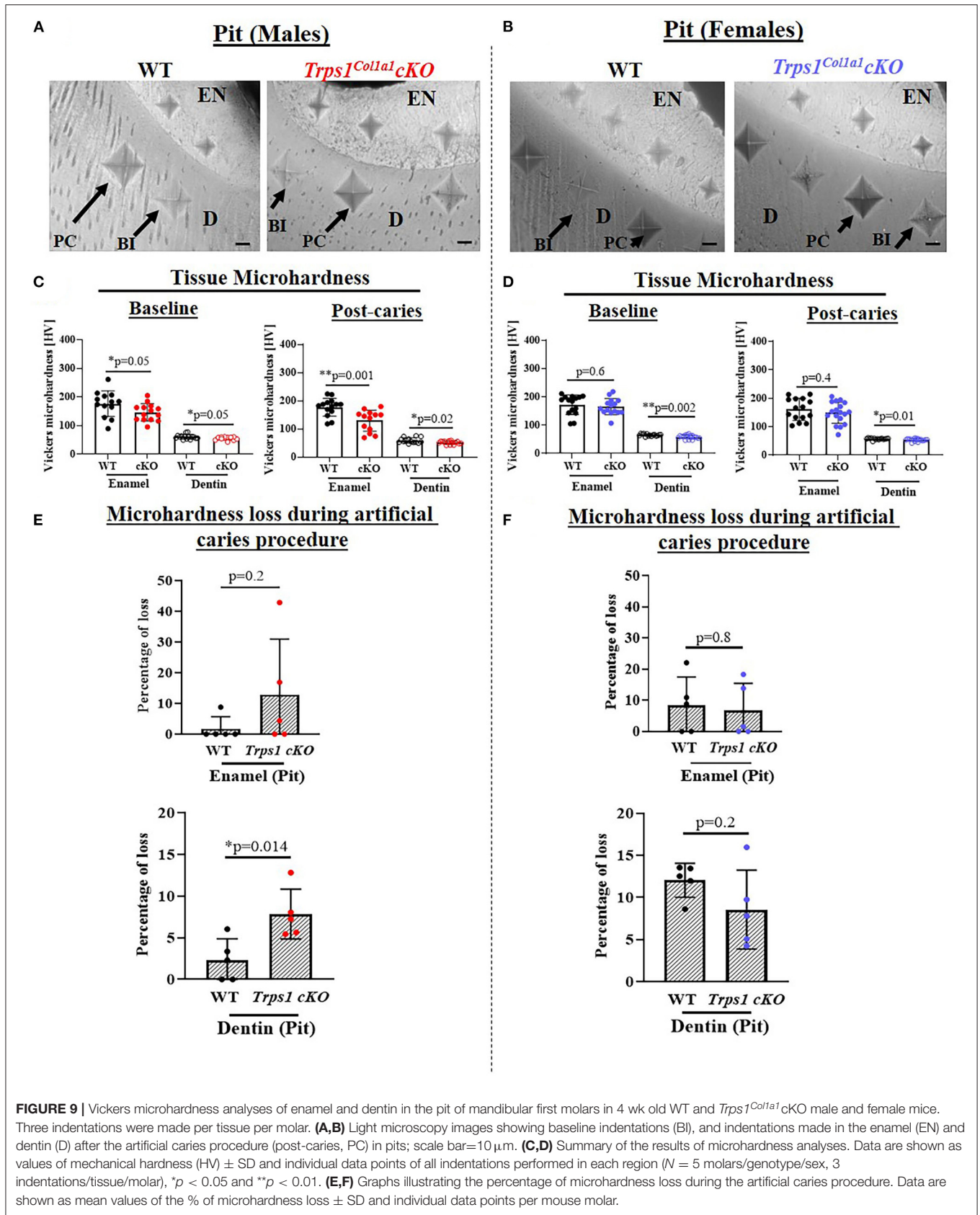


mechanisms. Specifically, we demonstrated that expression of several genes involved in phosphate homeostasis and hypophosphatemic rickets is regulated by *Trps1* in odontoblasts (47). Moreover, *Trps1* activity in cells producing mineralizing ECM changes depending on the status of the available extracellular phosphate (83), which also may contribute to the dentin mineralization problems in *Trps1<sup>Coll1a1</sup>cKO* mice. *Trps1* was also shown to repress expression of the *Dspp* gene coding for the major ECM protein of dentin (46), while the *Trps1* deficiency in 17IIA11 odontoblast cell line significantly impaired ability of these cells to mineralize, which was accompanied

by downregulation of multiple mineralization-related genes (47). Hence, it is likely that impaired dentin formation in *Trps1<sup>Coll1a1</sup>cKO* mice is caused by dysregulated transcription in odontoblasts that affects multiple genes crucial for the formation of sound dentin.

Interestingly, *Phex* is one of the phosphate homeostasis genes regulated by *Trps1* in odontoblasts and is also mutated in the most common form of the hypophosphatemic rickets, the X-linked hypophosphatemic rickets (47, 80, 84, 85). Hypophosphatemia caused by *PHEX* mutation results in a severely disrupted formation of circumpulpal dentin, while





**TABLE 1** | Summary of Vickers microhardness analyses and effect of artificial caries procedure on tissue microhardness loss in WT and *Trps1<sup>Coll1a1</sup>* cKO mandibular first molars.

	Region	Baseline [HV]		Post-Artificial Caries [HV]		% Microhardness loss	
		WT	<i>Trps1<sup>Coll1a1</sup></i> cKO	WT	<i>Trps1<sup>Coll1a1</sup></i> cKO	WT	<i>Trps1<sup>Coll1a1</sup></i> cKO
Males	Outer Enamel (OE)	<b>263.3 ± 16.4</b>	<b>208.8 ± 27.6*</b>	<b>251.0 ± 28.5</b>	<b>181.5 ± 27.6**</b> ( <sup>†††</sup> )	<b>5.8 ± 5.1</b>	<b>13.3 ± 3.2*</b>
	Inner Enamel (IE)	175.5 ± 14.2	179.3 ± 22.6	178.1 ± 18.8	178.3 ± 22.5	1.1 ± 1.6	1.2 ± 2.0
	Mantle Dentin (MD)	59.7 ± 7.7	52.0 ± 3.9	<b>52.5 ± 4.4(<sup>†</sup>)</b>	<b>46.6 ± 2.9* (<sup>††</sup>)</b>	11.7 ± 5.5	10.2 ± 2.7
	Circumpulpal Dentin (CPD)	<b>52.9 ± 2.5</b>	<b>45.6 ± 4.6*</b>	<b>49.8 ± 2.7(<sup>†</sup>)</b>	<b>38.9 ± 2.0*** (<sup>†</sup>)</b>	<b>5.9 ± 2.4</b>	<b>14.2 ± 7.2*</b>
	Enamel (Pit)	<b>180.6 ± 19.7</b>	<b>147.2 ± 18.3*</b>	<b>186.5 ± 7.8</b>	<b>130.7 ± 30.5**</b>	2.2 ± 4.4	12.8 ± 18.2
	Dentin (Pit)	60.5 ± 9.2	55.8 ± 5.3	59.7 ± 10.4	51.5 ± 5.8 ( <sup>††</sup> )	<b>2.3 ± 2.9</b>	<b>7.8 ± 3.0*</b>
Females	Outer Enamel (OE)	<b>250.7 ± 22.8</b>	<b>204.4 ± 20.8*</b>	<b>248.9 ± 17.1</b>	<b>193.1 ± 27.6** (<sup>†</sup>)</b>	3.0 ± 4.7	5.9 ± 4.7
	Inner Enamel (IE)	177.6 ± 15.8	173.1 ± 16.1	191.9 ± 18.7	169.3 ± 33.4	<b>0.0</b>	<b>8.1 ± 7.5*</b>
	Mantle Dentin (MD)	54.0 ± 5.8	52.6 ± 3.3	49.3 ± 4.3( <sup>†</sup> )	48.0 ± 2.5 ( <sup>†</sup> )	8.6 ± 4.2	8.4 ± 5.8
	Circumpulpal Dentin (CPD)	<b>54.2 ± 5.5</b>	<b>47.2 ± 3.0*</b>	<b>49.5 ± 4.9(<sup>††</sup>)</b>	<b>40.0 ± 4.4* (<sup>†</sup>)</b>	8.7 ± 2.0	15.2 ± 8.3
	Enamel (Pit)	172.1 ± 24.4	153.6 ± 33.4	161.1 ± 39.2	146.6 ± 37.3	8.4 ± 9.13	6.7 ± 8.7
	Dentin (Pit)	65.1 ± 3.0	58.1 ± 6.8	57.3 ± 2.7( <sup>†††</sup> )	52.9 ± 4.7 ( <sup>†</sup> )	12.0 ± 2.0	8.6 ± 4.7

The data are expressed as the mean values ± SD (N = 5 mice per group; for each mouse the mean value of 3 indentations in each tissue was used). Mechanical hardness values and the percentage of microhardness loss during the artificial caries procedure were compared between WT and *Trps1<sup>Coll1a1</sup>* cKO using an unpaired t-test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. Bold numbers highlight statistical significant differences between WT and *Trps1<sup>Coll1a1</sup>* cKO. Comparison (paired t-test) between baseline and post-artificial caries [HV] of outer enamel (OE), inner enamel (IE), mantle (M), and circumpulpal dentin (CPD) /genotype/sex, <sup>†</sup>p < 0.05, <sup>††</sup>p < 0.01, <sup>†††</sup>p < 0.001.

mantle dentin is unaffected. Since, all of these dental pathologies are present in *Trps1<sup>Coll1a1</sup>* cKO mice, affecting especially males; this suggests that differences between dental phenotypes of *Trps1<sup>Coll1a1</sup>* cKO males and females might be in part due to dysregulation of the *Phex* gene, which is located on the X-chromosome. Another possible explanation of the differences between *Trps1<sup>Coll1a1</sup>* cKO males and females is that the *Trps1* itself has been reported to interact with sex hormones such as androgen and estrogen (86–88). The interaction of *Trps1* with sex hormones is quite complex and occurs at multiple levels. For example, human prostate cancer studies demonstrated that *TRPS1* is repressed by androgens. On the other hand, *TRPS1* can inhibit androgen receptor signaling driving genes such as prostate-specific antigen and promote apoptosis in the absence of androgen (86, 89, 90). *TRPS1* has been also shown to regulate estrogen receptor binding to regulatory DNA elements, in part by modulation of chromatin transcriptional availability (91). The reported here differences between *Trps1<sup>Coll1a1</sup>* cKO male and female dental mineralized tissues are consistent with the previous reports that *Trps1* differentially modulates the bone mineral density between male and female mice (92). This set of evidence may explain in part, why 4 wk old *Trps1<sup>Coll1a1</sup>* cKO males (during onset of puberty), present with a stronger dental phenotype compared to females.

Among other dental findings in *Trps1<sup>Coll1a1</sup>* cKO mice relevant to the predisposition to dental caries is malocclusion. Dental caries is a common complication of malocclusion

(93). This abnormality, presented as misalignment of the teeth relative to the body of a mandible, was detected in both *Trps1<sup>Coll1a1</sup>* cKO males and females. This and severe root exposure detected in *Trps1<sup>Coll1a1</sup>* cKO mice suggest impaired formation of the alveolar bone, which is caused most likely by deficiency of *Trps1* in osteoblasts, as osteoblasts are another cell type expressing Cre from the *2.3kbColl1a1* promoter (54). Notably, severe tooth misalignments not only increase risk of dental caries but also of periodontal disease (94), underlining the role of *Trps1* not only in formation of sound mineralized dental tissues, but also in the dento-alveolar complex.

In summary, the compromised quality of the tooth mineralized tissues, expressed as softer and less acid-resistant enamel and dentin, together with decreased dentin layer, tooth misalignment, localized tooth mineralization defects in occlusal and buccal pits detected in *Trps1<sup>Coll1a1</sup>* cKO mice suggests that *TRPS* patients are genetically predisposed to dental caries. Hence, *TRPS* patients may benefit from more assertive prevention strategies and early interventions to mitigate dental caries risk and improve oral health.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC protocol #19095648).

## AUTHOR CONTRIBUTIONS

MS, EB, and DN: conception and design of the study. MS ( $\mu$ CT histology, microindentation assay, artificial dental caries procedure, and tamoxifen administration to mice), DN (generation of cKO mice), PH, KV, and LL ( $\mu$ CT), CR (histology, genotyping) data acquisition. MS, PH, LL, EB, and DN contributed to the data analyses. MS and DN wrote the manuscript with contribution from all the authors. All authors of the study have met authorship criteria established by the International Committee of Medical Journal Editors statement of Uniform Requirements for Manuscripts submitted to Biomedical Journals. In that regard, all co-authors are responsible for the reported research and have approved the final version of the manuscript submitted.

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## SUPPLEMENTARY MATERIAL

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