

Original Research

SCPPPQ1 Promotes Ameloblast Mineralization via the TGF- β 1 Signaling Pathway

Wenyu Guo¹, Zhenzhen Xu¹, Yumin Wang², Haiyu Mu³, Qing Chu¹, Yanying Tian¹, Guolei Zhu¹, Lili Xiang^{1,*}, Yuguang Gao^{1,*}

¹Department of Pediatrics and Preventive Dentistry, Binzhou Medical University Hospital, 256600 Binzhou, Shandong, China

²Institute of Stomatology, Binzhou Medical University, 264003 Yantai, Shandong, China

³Department of Stomatology, Yantai Yuhuangding Hospital, Qingdao University, 264001 Yantai, Shandong, China

*Correspondence: tianyahajiao.2012@163.com (Lili Xiang); gaoyuguang@bzmc.edu.cn (Yuguang Gao)

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Abstract

Background: Secretory Calcium-Binding Phosphoprotein Proline-Glutamine Rich 1 (SCPPPQ1) has been recognized as a critical contributor to tooth development; however, its precise molecular function in enamel formation remains poorly defined. **Methods:** To explore the regulatory mechanisms of SCPQQ1, we employed *in vivo* and *in vitro* models utilizing an SCPQQ1-knockout mouse model and ameloblast-like cell (ALC) line with stable SCPQQ1 overexpression. Using histology and stereomicroscopy, we found that SCPQQ1 deficiency results in severe enamel hypomineralization and structural defects, proving it is essential for enamel maturation. Meanwhile, SCPQQ1 overexpression in ALCs significantly boosted mineral deposition and upregulated critical differentiation markers, including Runx2 and alkaline phosphatase (ALP). Mechanistically, we showed that these pro-amelogenic effects rely on the TGF- β 1 signaling pathway; specifically, the TGF- β 1 pathway inhibitor SIS3 successfully blocked the mineralization induced by SCPQQ1. **Conclusions:** Together, our results demonstrated SCPQQ1 as a crucial positive regulator of enamel formation via the TGF- β 1–Runx2/ALP signaling axis. These findings uncover an important molecular node in tooth development and highlight the therapeutic potential of targeting SCPQQ1 for enamel repair and regeneration.

Keywords: ameloblasts; mineralization; TGF- β 1 signaling pathway

1. Introduction

Although tooth enamel is the most highly mineralized tissue in the human body, it lacks regenerative capacity once damaged because of its acellular structure [1]. Amelogenesis is a strictly timed and spatially organized process, designed to build an enamel matrix strong enough to withstand immense chewing forces [2]. When this process goes awry—such as in enamel hypomineralization—it results in qualitative defects characterized by reduced mineral content, discoloration, and increased susceptibility to wear and decay [3]. Such defects are often linked to dysregulation of ameloblasts during the maturation stage of enamel formation [4]. At this point, ameloblasts drive crystal thickening and enamel hardening by efficiently degrading and clearing out matrix proteins, while simultaneously pumping massive amounts of ions into the matrix. Once the cells lay down a laminin-332-enriched specialized basal lamina (sBL) on the enamel surface, they recruit key proteins to the site. High levels of Amelotin (AMTN), Odonogenic Ameloblast-Associated Protein (ODAM), and secretory calcium-binding phosphoprotein proline-glutamine rich 1 (SCPPPQ1) assemble into a supramolecular complex. This complex acts as a vital bridge, securing the maturation-stage ameloblasts firmly to the underlying mineralized enamel [5,6].

Located within the secretory calcium-binding phosphoprotein (SCPP) gene cluster and encoded as a 9-kDa protein [7,8], SCPQQ1 contains a high proportion of hydrophobic residues—proline, leucine, and phenylalanine accounting for over 40% [9]. Like many SCPP family members essential for vertebrate hard tissue formation [10,11,12], SCPQQ1 is highly conserved across species and is primarily expressed by ameloblasts during the late maturation stage of amelogenesis [7]. Its specific spatiotemporal expression pattern suggests a specialized function in the final hardening of enamel. Previous studies have shown that SCPQQ1 binds tightly to hydroxyapatite (HA)—the primary mineral in enamel, and teams up with AMTN and ODA to anchor ameloblasts to the mineralizing surface [1,5]. Overall, these findings imply that SCPQQ1 is an indispensable structural and adhesive player in enamel development.

The production of functional proteins like SCPQQ1 is tied to broader signaling cascades that govern ameloblast differentiation and enamel biomineralization [13]. A central regulator in this process is the TGF- β 1 pathway, as TGF- β 1, which is expressed throughout amelogenesis, plays multifaceted roles, including regulating cell proliferation, gene expression, ion transport, and apoptosis during the maturation stage [14,15,16,17]. Its critical na-



ture is further emphasized by the fact that knocking out its downstream effector, Smad3, leads to severe enamel hypomineralization, establishing Smad3 as essential for enamel biomineralization [18]. Furthermore, the TGF- β 1 axis acts upstream of Runx2, a master transcription factor for hard tissue formation. Runx2 not only drives the lineage commitment of mesenchymal progenitors but also directly switches on a suite of genes crucial for matrix synthesis and mineralization [19]. Yet, despite SCPPPQ1's known contribution to enamel development, it remains entirely unknown whether this specific protein actively participates in the TGF- β 1 signaling pathway.

In this study, we set out to map the functional landscape of SCPPPQ1 during enamel formation. To achieve this, we used a dual approach, combining an *in vivo* SCPPPQ1-null mouse model with *in vitro* SCPPPQ1-overexpressing ameloblast-like cells. Our findings reveal that SCPPPQ1 does more than just act as a structural bridge; it is required to modulate the TGF- β 1 signaling cascade to actively drive mineralization. Ultimately, these insights refine our current understanding of amelogenesis and point to SCPPPQ1 as a promising molecular target for treating structural enamel defects.

2. Materials and Methods

2.1 Animal Studies

We used postnatal day 15 (P15) male C57BL/6 mice weighing approximately 5–7 g. The study included two genotypes: wild-type (WT) and SCPPPQ1 knockout (KO), with six mice per group. All procedures complied with the ARRIVE guidelines and the National Research Council's Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Animal Ethics Committee of Binzhou Medical University (Approval No. 2025-L029). We housed the mice in a climate-controlled room (22 ± 2 °C) with a standard 12-hour light/dark cycle and unrestricted access to food and water. On postnatal day 15, we deeply anesthetized the mice with 3% isoflurane and euthanized them using CO₂ asphyxiation (30%–40% concentration; 10%–30% chamber volume/min flow rate). We immediately dissected the mandibles, fixed them overnight at 4 °C in 4% paraformaldehyde (PFA), and decalcified them with 10% EDTA (pH 7.4). After decalcification, we embedded the samples in paraffin and cut them into 5 μ m slices for histological analysis. No random allocation was performed. Animals were assigned to WT or SCPPPQ1-knockout groups according to their genotypes. No specific inclusion or exclusion criteria were established a priori. All animals (n = 6 per group) were successfully processed and included in the final analysis, and no animals or data points were excluded. Group-assignment information for all mice was known to the experimenter during animal allocation and routine experimental procedures. Blinding was implemented for histological outcome assessment: the operator measuring staining results was unaware of sample genotype

groups. Data analysis was performed using anonymised sample numbers to minimise bias.

2.2 Histological Analysis

The 5 μ m paraffin sections were first deparaffinized using xylene and rehydrated through a series of graded ethanols. To prepare for immunohistochemistry (IHC), antigens were retrieved in a citrate buffer heated to 90 °C for 30 minutes. After quenching endogenous peroxidase activity with 3% H₂O₂ and blocking the tissues with goat serum, the slides were incubated overnight at 4 °C with primary antibodies targeting TGF- β 1 and Smad3. Next, the sections were treated with biotinylated secondary antibodies and a streptavidin-HRP complex (ABC kit). Signal visualization was achieved using a DAB substrate kit, followed by a standard nuclear counterstain. Negative controls were established in parallel by simply omitting the primary antibody. Finally, the slides were dehydrated, cleared, and mounted for observation under a microscope. For quantitative analysis of IHC staining, images were analyzed using ImageJ software. Regions of interest (ROIs) were selected within the ameloblast layer. Three randomly selected fields were analyzed for each sample. Background correction was performed to remove non-specific staining signals, and the quantified pixel values were normalized accordingly.

All micrographs were acquired using the same microscope settings throughout the study. The images presented are the best-quality raw files obtained from our samples, and no better-quality alternative image sets from these tissue sections are available.

2.3 Scanning Electron Microscopy (SEM)

2.3.1 Sample Preparation

Mandibles collected from P15 WT and KO mice were immediately fixed in 4% PFA for 12 hours and rinsed in PBS for another 12 hours. Under a stereomicroscope, the gingiva and surrounding soft tissues were carefully peeled away to expose the teeth without damaging the delicate enamel. The exposed mandibles were rinsed in distilled water for 10 minutes, briefly treated with 0.01 N NaOH for 1 minute, washed again in PBS for 12 hours, and finally dehydrated using a graded ethanol series (70%, 80%, 90%, and 95% for 20 minutes each, ending with 100% for 40 minutes).

2.3.2 SEM Analysis

To prepare for ultrastructural imaging, the dehydrated samples were wet-ground using a sequence of progressively finer abrasive papers (from 600 to 3000 grit). They were then polished for 20 minutes with 1500-grit paper submerged in a 1 N NaOH solution. After a brief rinse, the polished enamel surfaces were etched for 30 seconds with 37% phosphoric acid. Following a final wash, the mandibles were air-dried for 2 hours, sputter-coated with gold, and examined under a scanning electron microscope to evaluate the enamel rod morphology in the lower incisors.

2.4 Cell Culture and Overexpression Model

A murine ameloblast-like cell line (ALC), a generous gift from Prof. T. Sugiyama (Akita University, Japan), was grown in DMEM/F12 medium. This was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and maintained in a 37 °C incubator with a 5% CO₂ atmosphere. To establish an SCPPPQ1-overexpressing model, these ALCs were transduced with a lentivirus carrying the SCPPPQ1 gene, while an empty vector was utilized for the negative control group. Prior to the experiments, all cell lines were validated via short tandem repeat (STR) profiling, and tested negative for mycoplasma. TGF-β1 and SIS3 were purchased from Abbkine and Meilunbio (China), with catalog numbers PRP1171 and MC9623-1, respectively.

2.5 Lentiviral Transduction

To find the ideal multiplicity of infection (MOI), ALCs (between passages 3 and 6) were plated in 96-well plates. Once they reached roughly 40% confluency, they were exposed to an empty control lentivirus at various MOIs, aided by 5 µg/mL Polybrene. The necessary viral volume was calculated using the standard formula: Volume (µL) = [(Desired MOI × Cell Number) / Viral Titer (TU/mL)] × 1000. After 72 hours, GFP expression was checked under a fluorescence microscope to gauge transduction efficiency, revealing that an MOI of 100 was optimal for future experiments. To build stable cell lines, ALCs in 24-well plates were transduced at this MOI of 100 with either the target or control lentivirus (LV5-NC). The viral medium was swapped for fresh complete medium 24 hours later. Based on a prior kill curve that identified 1 µg/mL as the cytotoxic dose for untransduced cells, a 14-day puromycin selection (1 µg/mL) was applied. The selection medium was refreshed every two days, and the surviving colonies were successfully isolated and expanded.

2.6 Mineralization Induction and Staining

For the mineralization assays, cells plated in 6-well plates were switched to an induction medium containing standard growth medium, 10 nM dexamethasone, 50 µg/mL ascorbic acid, and 10 mM β-glycerophosphate. This medium was replaced every other day. On days 3, 7, and 14, the cells were washed with PBS and fixed in 4% PFA. Alkaline phosphatase (ALP) activity was measured using a standard BCIP/NBT staining kit. To evaluate mineralized matrix formation, the fixed cells were stained with a 1% Alizarin Red S (ARS) solution at 37 °C for 30 minutes. Excess dye was thoroughly washed away before imaging. For quantification, the bound ARS stain was eluted using 10% cetylpyridinium chloride, and the resulting absorbance was read on a microplate reader. For molecular analysis of TGF-β1 and Smad3 expression, cells were collected after 7 days of mineralization induction for subsequent RT-qPCR and Western blot analyses. For rescue experiments, gradi-

ent concentrations of TGF-β1 (0, 0.5, 1 ng/mL) and SIS3 (0, 1, 5 nmol/mL) were supplemented into the mineralization induction medium throughout the 14-day induction period. The corresponding reagents were freshly added each time the medium was changed.

2.7 Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted from the cell cultures using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). To ensure genomic DNA was fully eliminated before reverse transcription, cDNA was synthesized utilizing a kit containing gDNA Eraser. The qPCR reactions were then run on a LightCycler® 96 system (Roche) with the SYBR Green Pro Taq HS qPCR mix. Data were analyzed using the standard $2^{-\Delta\Delta C_t}$ method [20,21], utilizing GAPDH as the internal control. ALC cells were treated with gradient concentrations of TGF-β1 (0, 0.5, 1 ng/mL) and SIS3 (0, 1, 5 nmol/mL). Cells were incubated with reagents for 6 h prior to sample collection for RT-qPCR analysis.

2.8 Western Blot Analysis

ALC cells were treated with gradient concentrations of TGF-β1 (0, 0.5, 1 ng/mL) and SIS3 (0, 1, 5 nmol/mL). Cells were incubated with reagents for 6 h prior to sample collection for Western blot analysis. Cells were lysed in RIPA buffer to extract total protein, and the concentrations were measured using a BCA assay kit. Equal protein loads were separated via SDS-PAGE and transferred onto PVDF membranes. These membranes were blocked in 5% non-fat milk before being probed overnight at 4 °C with primary antibodies targeting Runx2, ALP, TGF-β1, Smad3, and GAPDH. After a series of washes, the blots were incubated with HRP-conjugated secondary antibodies. Finally, the protein bands were illuminated with an ECL substrate and captured using a Tanon imaging system. The antibodies used are listed as below: Anti-Runx2 (Bio-swamp, PAB33483, 1:1000), Anti-ALP (Bio-swamp, RMAB50712, 1:1000), Anti-phospho-Smad3 (P-Smad3) (Bio-swamp, RMAB53435, 1:1000), Anti-Smad3 (total Smad3) (Bio-swamp, RMAB48789, 1:1000), Anti-TGF-β1 (Bio-swamp, PAB59327, 1:1000), and Anti-GAPDH (Bio-swamp, MAB45855, 1:5000).

Band intensities were measured by standard densitometry and normalized to the corresponding loading controls. Minor visual variations in band appearance may arise from figure-layout formatting, while the quantitative densitometry results remain unchanged.

2.9 Statistical Analysis

All numerical data are reported as the mean ± standard deviation (SD) based on a minimum of three independent replicates. Statistical evaluations were carried out in GraphPad Prism 10. Group differences were analyzed using either a Student's *t*-test or a one-way ANOVA, with a *p*-value below 0.05 deemed statistically significant (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

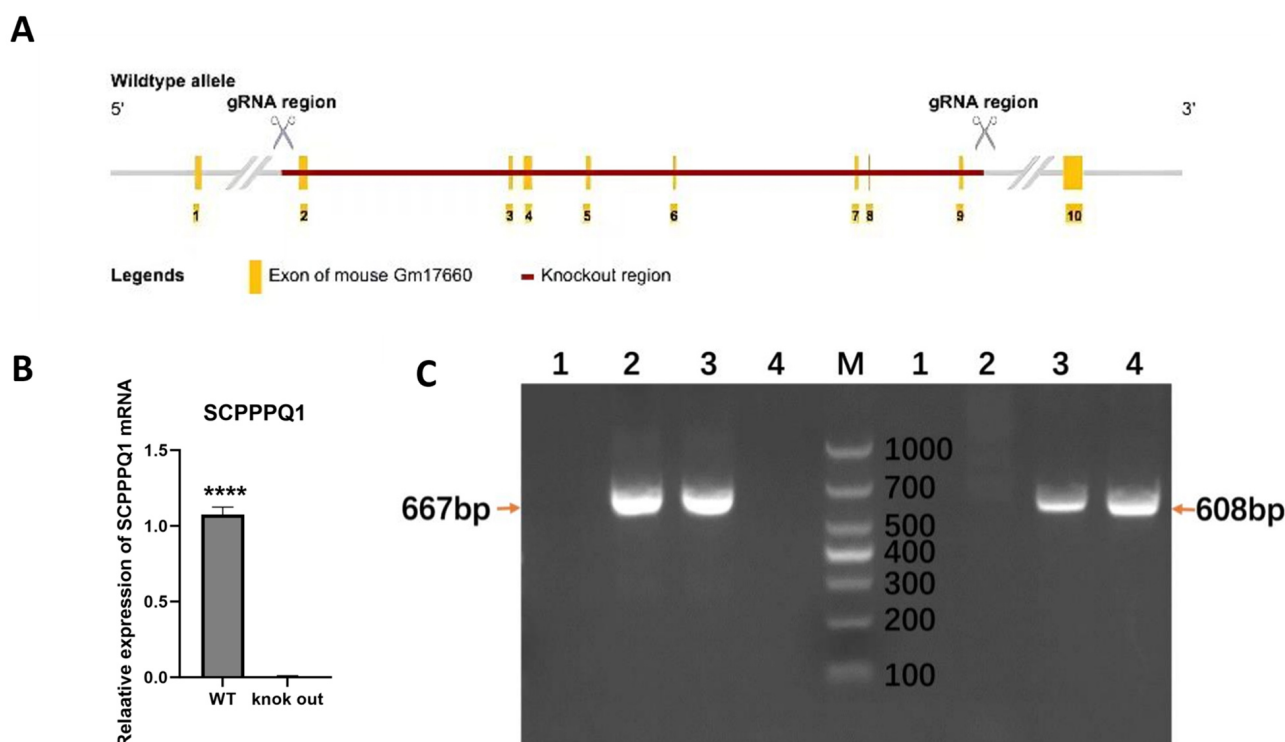


Fig. 1. Generation and validation of the SCPPPQ1 KO mouse model. (A) Schematic diagram outlining the CRISPR/Cas9 targeting strategy used to create the KO mice. (B) RT-qPCR analysis confirming the loss of SCPPPQ1 mRNA expression in the knockout group. (C) A representative agarose gel showing the PCR genotyping results for the offspring. ‘M’ stands for the DNA marker. The bands flanking the marker represent the PCR products amplified using primers F1/R1 (667 bp) and F2/R1 (608 bp). Lane assignments are as follows: Lane 1, distilled water negative control (Blank); Lane 2, SCPPPQ1 homozygous knockout (KO); Lane 3, heterozygote (Het); Lane 4, wild-type (WT) (n = 6). SCPPPQ1, secretory calcium-binding phosphoprotein proline-glutamine rich 1; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas9, CRISPR-associated protein 9; RT-qPCR, real-time quantitative PCR; gDNA, genomic DNA. **** $p < 0.0001$.

3. Results

3.1 SCPPPQ1 Deficiency Impairs Enamel Mineralization in Mice

We generated the SCPPPQ1 KO mouse model using CRISPR/Cas9 genome engineering (Protocol No.: KOA1220406DX5) [22]. As detailed in our construction strategy (Fig. 1A), the SCPPPQ1 gene (Gm17660; NM_001163772; ENSMUSG00000091034) sits on mouse chromosome 5 and contains 10 exons. Because the coding sequence runs from exon 2 to exon 9, we specifically targeted this entire segment for deletion to completely abolish the gene’s function. To achieve this, we co-injected Cas9 mRNA alongside four specific guide RNAs (gRNA-A1, A2, B1, and B2) into fertilized mouse zygotes to produce our founder mice. We then used PCR genotyping on the offspring to successfully confirm the SCPPPQ1 KO mice (Fig. 1B,C).

To figure out the physiological role of SCPPPQ1, we examined the incisors of our KO mice at the tooth eruption stage under a stereomicroscope, and found that the KO mice suffered from severe enamel wear—a defect com-

pletely missing in their WT littermates (Fig. 2A). In line with these findings, SEM imaging showed that the KO molars were rough and heavily worn down, a stark contrast to the smooth, fully mineralized surface seen in WT mice (Fig. 2B). On top of that, the enamel of KO incisors looked porous and disorganized at the micro-level, lacking the highly ordered rod structure typical of healthy enamel (Fig. 2C). Taken together, these findings suggest that SCPPPQ1 plays an essential role in proper enamel mineralization *in vivo*.

3.2 SCPPPQ1 Promotes Mineralization in Ameloblast-Like Cells

To explore the cellular mechanisms driving our *in vivo* observations, we generated an ALC line that stably overexpresses SCPPPQ1 via lentiviral transduction (Fig. 3A). After using a CCK-8 cytotoxicity assay to pinpoint 1 $\mu\text{g/mL}$ as the optimal concentration for cell selection (Fig. 3B), we verified the robust overexpression of SCPPPQ1 through both GFP fluorescence after lentiviral transduction and RT-qPCR (Fig. 3C,D).

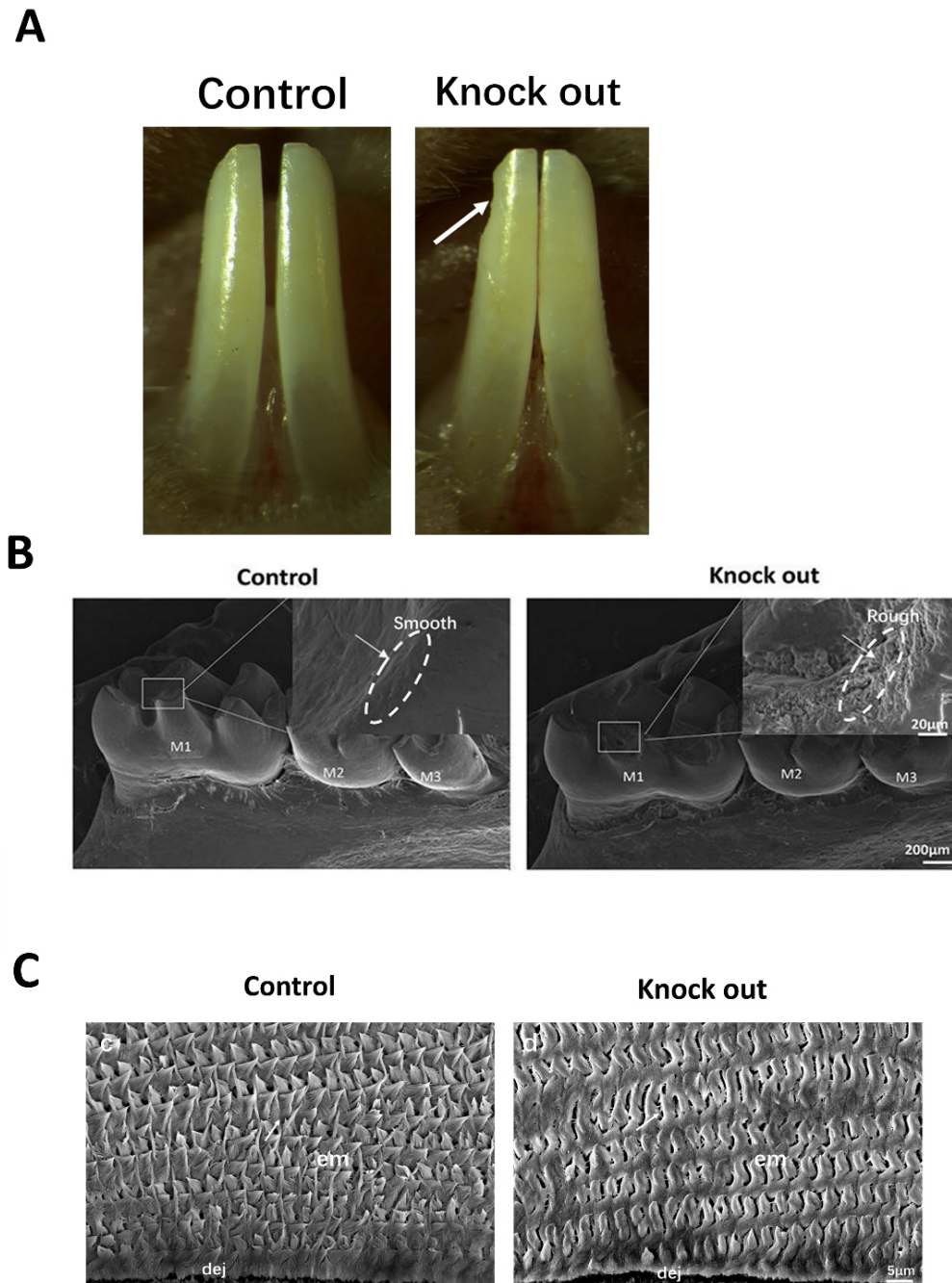


Fig. 2. *SCPPPQ1* KO leads to defective enamel mineralization. (A) Stereomicroscopic images highlighting distinct wear on the incisor edges of KO mice, a feature notably absent in WT controls (white arrows). (B) SEM images of the first molar enamel surface. WT mice present a smooth, intact surface, which stands in sharp contrast to the rough and abraded texture observed in KO mice. Scale bars: 200 μm (main images) and 20 μm (magnified images). (C) High-magnification SEM imaging (lower panel, 500 $\times$) of the incisors. While WT mice demonstrate a neatly organized enamel prism structure, the KO enamel displays a porous and completely disordered arrangement ($n = 6$). Scale bar: 5 μm . Abbreviations: M1, first molar; M2, second molar; M3, third molar; am, ameloblasts; de, dentin; em, enamel matrix; SEM, scanning electron microscopy; dej, dentinoenamel junction.

Next, we evaluated the functional impact of SCPPPQ1 on the cells' mineralization capacity. During the induction time course, the SCPPPQ1-overexpressing (OE) cells

displayed noticeably stronger staining for ALP—a classic early marker of mineralization than the negative controls at days 7 and 14, but not at day 3 (Fig. 4A,B). This enhanced

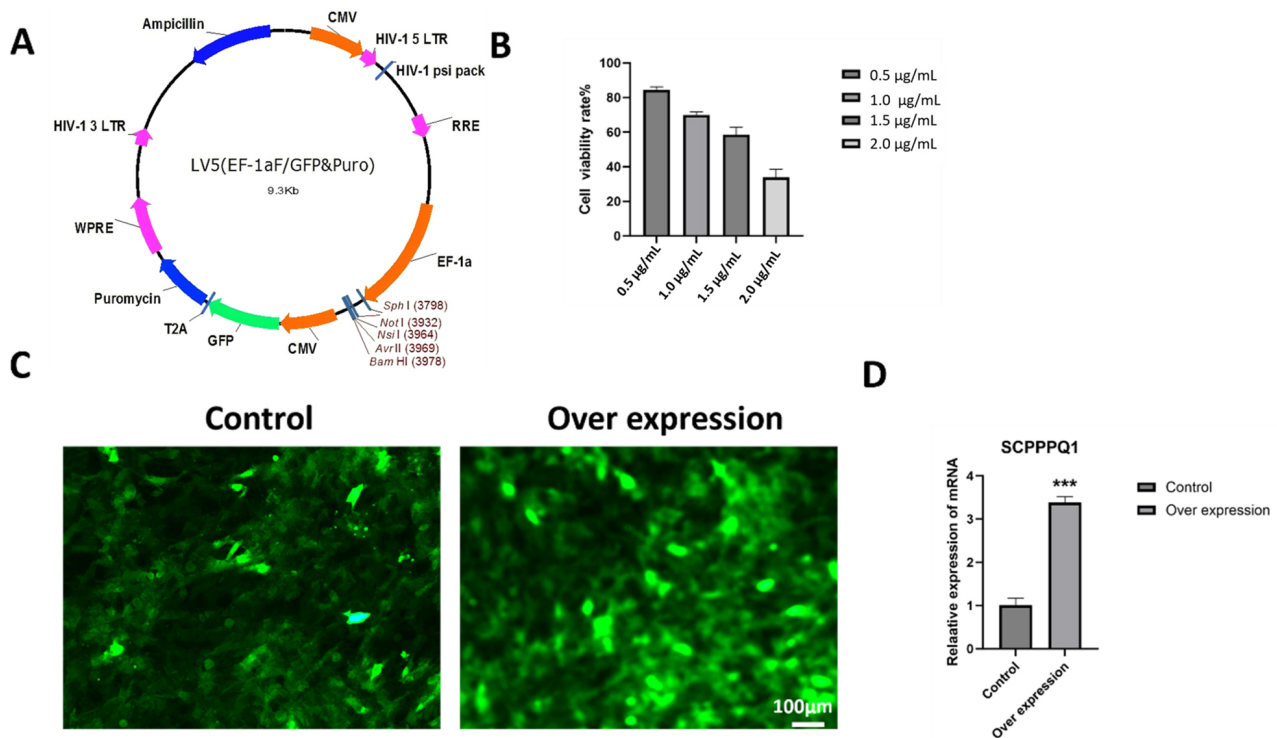


Fig. 3. Generation and validation of the SCPPPQ1-overexpressing cell line. (A) Schematic of the lentiviral vector plasmid used for transduction. (B) CCK-8 assay results determining the optimal drug concentration for stable cell selection. (C) Fluorescence microscopy (100× magnification) confirming high transduction efficiency 72 hours after lentiviral infection. (D) RT-qPCR analysis validating the successful overexpression of SCPPPQ1 at the mRNA level. Scale bar = 100 µm. CCK-8, Cell Counting Kit-8; HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; RRE, Rev response element; LV5, lentiviral vector 5; GFP, green fluorescent protein; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element; CMV, cytomegalovirus; EF-1α, elongation factor 1 alpha; Puro, puromycin; T2A, Thosea asigna virus 2A peptide; SphI, SphI restriction enzyme; NotI, NotI restriction enzyme; NsiI, NsiI restriction enzyme; AvrII, AvrII restriction enzyme; BamHI, BamHI restriction enzyme. *** $p < 0.001$.

mineralization potential was further corroborated by ARS staining, which highlighted a striking increase in mineralized nodule formation in the OE group at days 3, 7 and 14 (Fig. 4C,D).

To uncover the molecular basis for this boosted mineralization, we checked the expression of key downstream regulators. Both RT-qPCR and Western blot analyses confirmed that SCPPPQ1 overexpression markedly upregulates the mRNA and protein levels of Runx2 and ALP (Fig. 5A–D). Ultimately, these *in vitro* data suggest that SCPPPQ1 drives mineralization by activating these critical transcription factors and enzymes.

3.3 SCPPPQ1 Positively Regulates the TGF-β1 Signaling Pathway

To elucidate the potential mechanism governing the observed phenotype, we examined the status of the TGF-β1 pathway, a crucial regulator of amelogenesis. IHC revealed a striking drop in the expression of both TGF-β1 and its core downstream mediator, Smad3, within the ameloblasts of KO mice compared to their WT littermates (Fig. 6A,B). Importantly, we ensured the reliability of this visual data

by maintaining strictly identical imaging settings across all samples and confirming minimal background in negative controls.

Shifting to our *in vitro* model, overexpressing SCPPPQ1 in ALCs produced the exact opposite effect. After 7 days of mineralization induction, SCPPPQ1 overexpression triggered a robust upregulation of TGF-β1 and Smad3 at both the mRNA and protein levels (Fig. 7A–D). Taken together, these paired *in vivo* and *in vitro* data strongly suggest that SCPPPQ1 acts as a crucial positive regulator of the TGF-β1 signaling cascade during enamel mineralization.

3.4 SCPPPQ1 Mediates Ameloblast Mineralization via the TGF-β1 Signaling Pathway

To definitively map the hierarchical relationship between SCPPPQ1 and TGF-β1 signaling, we first assessed the direct impact of exogenous TGF-β1 on ALC differentiation. As expected, treating cells with TGF-β1 dose-dependently boosted both ALP activity and mineralized nodule formation (Fig. 8A). Furthermore, while this treatment markedly upregulated the osteogenic markers Runx2

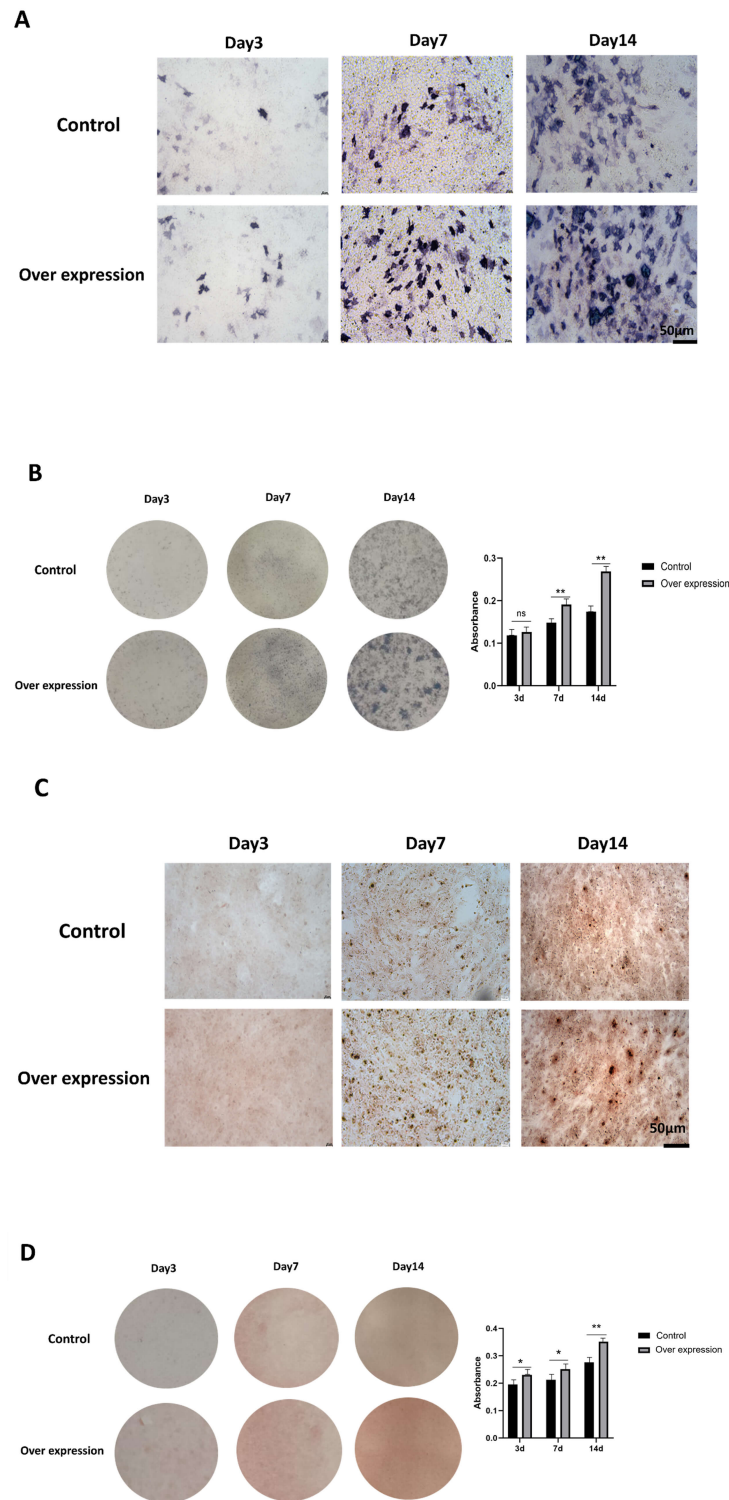


Fig. 4. SCPPPQ1 overexpression boosts the mineralization capacity of ameloblast-like cells (ALCs). For these assays, ALCs were stably transduced with either an empty vector (Negative Control, NC) or the SCPPPQ1-overexpression construct (OE), and then subjected to mineralization induction for up to 14 days. (A) Microscopic images tracking Alkaline Phosphatase (ALP) expression at days 3, 7, and 14. Scale bar: 50 μm. (B) Whole-well macroscopic views of the ALP staining paired with the quantitative readout of ALP activity. (C) Representative microscopy highlighting Alizarin Red S (ARS) staining, which reveals mineralized nodule formation at days 3, 7 and 14. Scale bar: 50 μm. (D) Macroscopic overviews of the ARS-stained plates alongside the quantitative analysis of total calcium deposition. “ns” denotes not significant; * $p < 0.05$; ** $p < 0.01$.

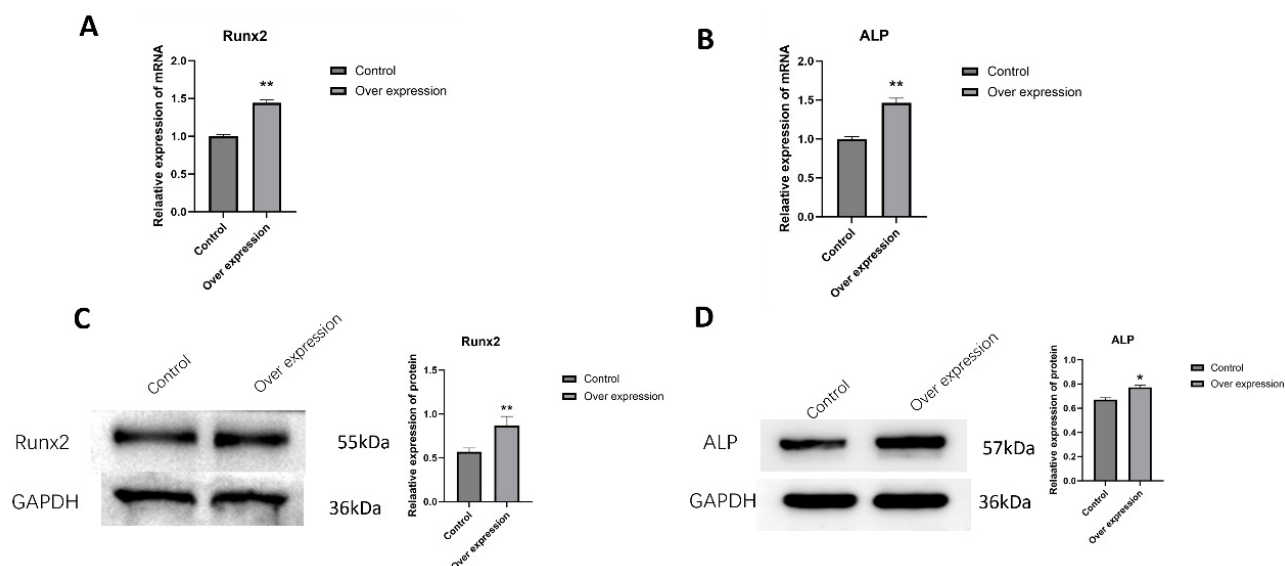


Fig. 5. SCPPPQ1 overexpression drives the upregulation of the key osteogenic markers Runx2 and ALP. (A,B) RT-qPCR analysis quantifying the elevated mRNA levels of Runx2 and ALP. (C,D) Western blot assays confirming corresponding increases in Runx2 and ALP at the protein level. Data are presented as the mean $\pm$ SD from three independent biological replicates. * $p < 0.05$, ** $p < 0.01$. SD, standard deviation.

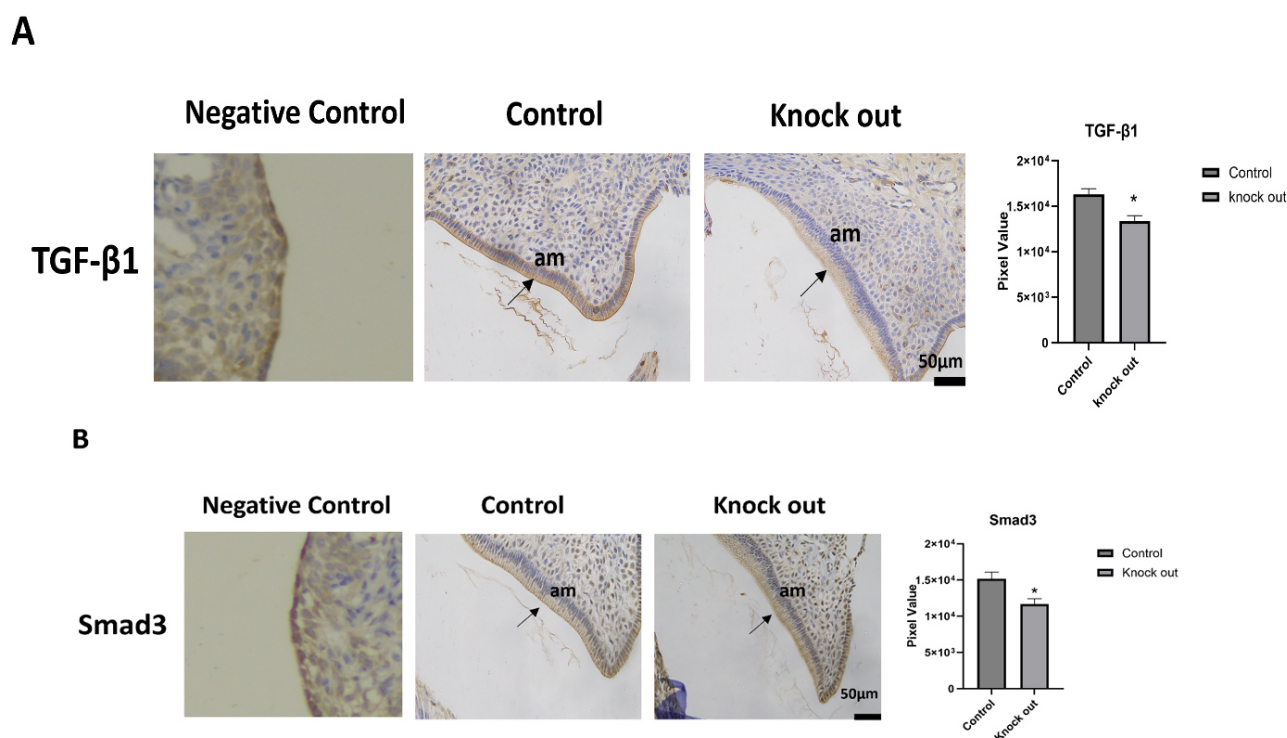


Fig. 6. SCPPPQ1 expression is tightly linked to the TGF- β 1 signaling pathway. (A,B) IHC staining highlighting the local expression of TGF- β 1 and Smad3 in the first molars of WT and KO mice ($n = 6$). A negative control section, processed without the primary antibody, is included to establish the baseline background. All micrographs were acquired with identical microscope settings. am, ameloblasts; IHC, immunohistochemistry; Smad3, SMAD family member 3. * $p < 0.05$.

and ALP, SCPPPQ1 expression remained essentially unchanged (Fig. 8B–G).

To prove that SCPPPQ1 relies on this specific pathway to function, we introduced SIS3, a well-characterized selec-

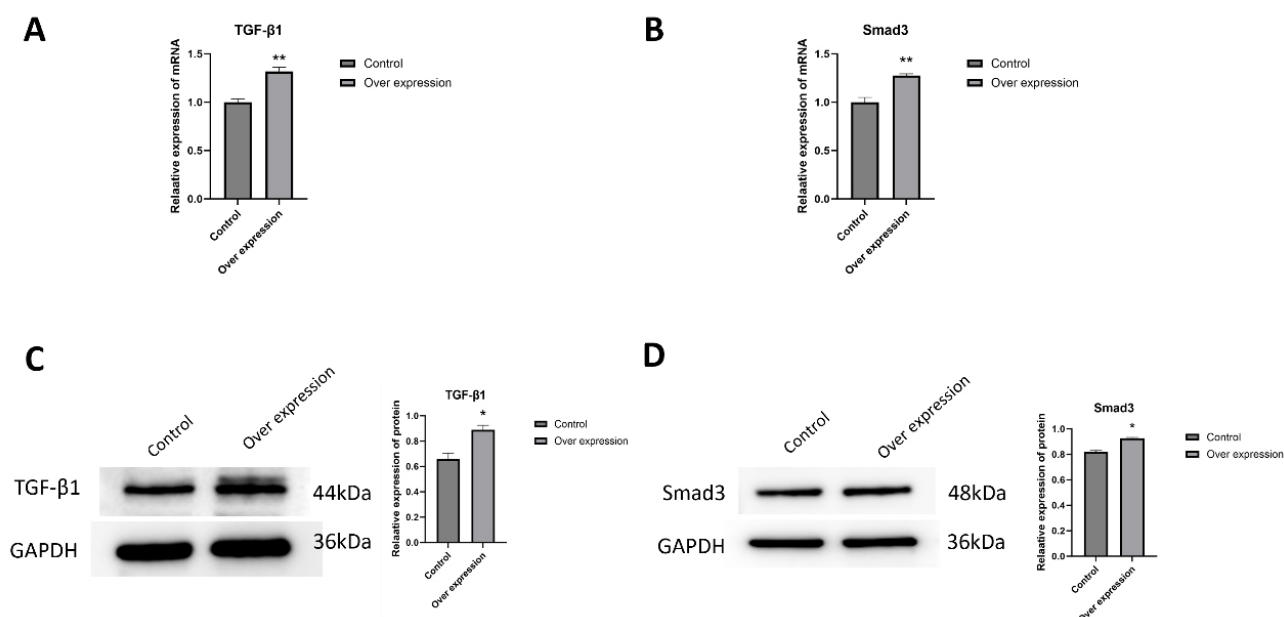


Fig. 7. SCPPPQ1 overexpression activates the TGF-β1 signaling pathway. (A,B) RT-qPCR data detailing the increased mRNA expression of TGF-β1 and Smad3 in SCPPPQ1-OE ALCs relative to the control group, following a 7-day mineralization induction. (C,D) Western blot results validating this trend at the protein level, showing clear upregulation of both TGF-β1 and Smad3 in the OE group. Data are presented as the mean $\pm$ SD, $n = 6$ biological replicates. * $p < 0.05$, ** $p < 0.01$.

tive inhibitor of Smad3 phosphorylation [23,24]. Although SCPPPQ1-OE cells typically display robust mineralization, SIS3 treatment severely abrogated this phenotype, pulling down both ALP activity and calcium deposition (Fig. 8H). At the molecular level, the inhibitor effectively nullified the SCPPPQ1-driven spikes in *Runx2* and *ALP* transcripts. Protein analysis confirmed the inhibitor's efficacy, showing a dose-dependent drop in Smad3 phosphorylation (measured by the P-Smad3 protein levels normalized to GAPDH) as SIS3 concentrations increased, effectively severing the signaling cascade (Fig. 8I,J). Ultimately, these rescue experiments demonstrated SCPPPQ1 drives ameloblast mineralization functionally through the activation of the TGF-β1/Smad3 signaling axis.

4. Discussion

Defective enamel mineralization remains a major clinical challenge, largely driven by the dysfunction of ameloblasts, the master architects of tooth enamel [25,26,27]. To deepen our molecular understanding of this condition, we set out to investigate SCPPPQ1. Although known to be uniquely expressed by ameloblasts during the late maturation stage [7], we identified it here as a key regulator with a previously unrecognized role in amelogenesis. Our *in vivo* data strongly suggested the physiological necessity of SCPPPQ1, as knocking it out in mice led to severe enamel mineralization defects. To tease apart the underlying mechanics, we then transitioned to an *in vitro* gain-of-function model. By stably overex-

pressing SCPPPQ1 in ameloblast-like cells (ALCs), we triggered a robust pro-mineralization program. This was evident through a time-dependent surge in ALP activity and calcium deposition, alongside a coordinated boost in both the mRNA and protein levels of *Runx2* and *ALP*. These results suggest that SCPPPQ1 is not just passively allowing mineral to deposit; rather, it actively kickstarts a molecular program highly similar to an osteogenic transcriptional module. Crucially, our data outline a signaling route downstream of SCPPPQ1. While adding exogenous TGF-β1 closely recapitulated the mineralization-promoting phenotype, applying the inhibitor SIS3 strongly blocked the effects of SCPPPQ1 overexpression. Together, these lines of evidence point to TGF-β1 signaling as the primary downstream bridge linking SCPPPQ1 to the activation of mineralization-related genes.

Collectively, these findings redefine the role of SCPPPQ1, upgrading it from a mere structural building block to an active, critical regulator of enamel development. Historically, SCPPPQ1 was known simply as a calcium-binding phosphoprotein found in maturation-stage ameloblasts, the basal lamina, and the junctional epithelium [5]. In that context, it serves as a cell-adhesion molecule, anchoring ameloblasts to the enamel matrix by teaming up with AMTN and ODAM [7]. Meanwhile, past studies have shown that lacking TGF-β1 disrupts the normal ebb and flow of enamel matrix proteins. Mechanistically, TGF-β1 co-localizes with *Runx2* in ameloblasts, steering enamel mineralization by controlling *Runx2* expression [28]. Here,

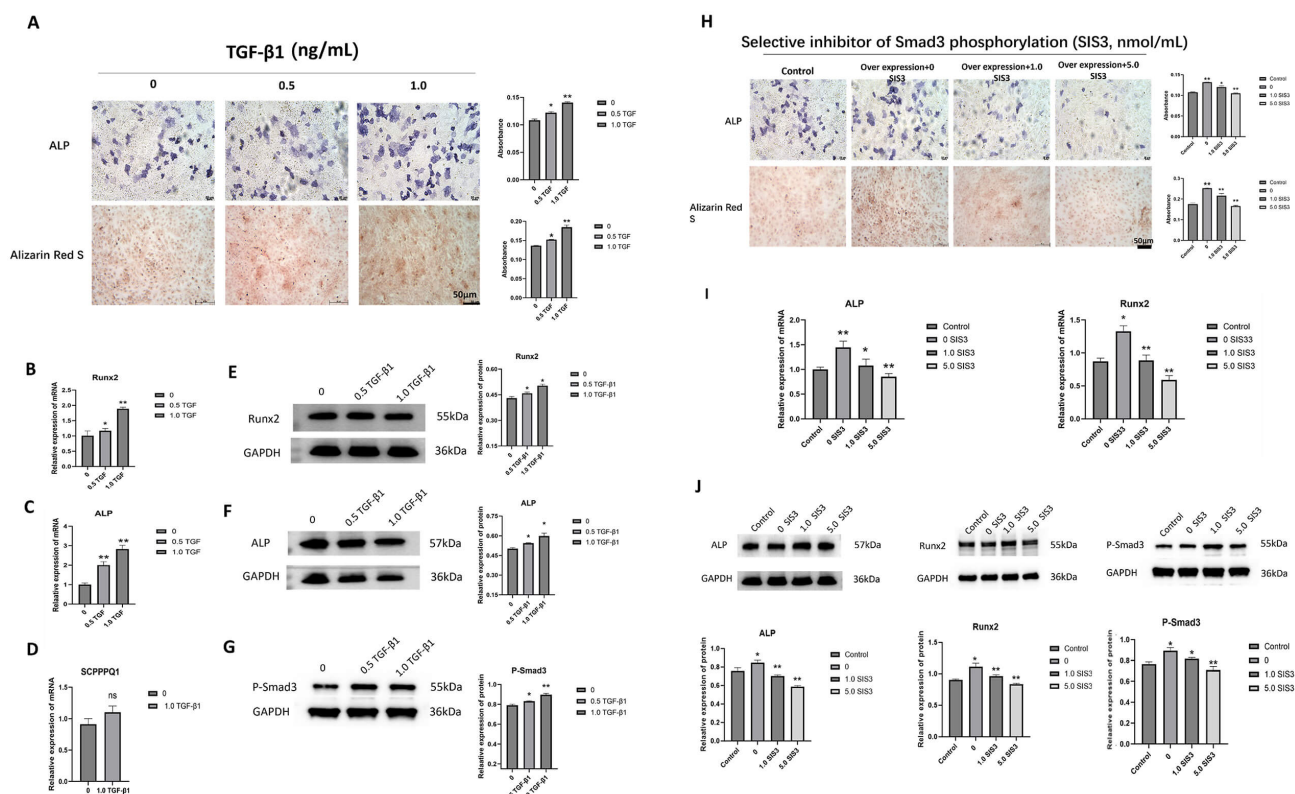


Fig. 8. SCPPPQ1 promotes ALC mineralization via the TGF- β 1 signaling pathway. (A) ALP and ARS staining, alongside corresponding quantification, in ALCs treated with a concentration gradient of exogenous TGF- β 1 for 14 days. Scale bar = 50 μ m. (B–G) RT-qPCR and Western blot analyses evaluating the expression of Runx2, ALP, and SCPPPQ1 in response to TGF- β 1 stimulation. (H) Rescue assays evaluating mineralization (via ALP and ARS staining) in NC and SCPPPQ1-OE cells treated with escalating doses of the Smad3 inhibitor, SIS3. Scale bar = 50 μ m. (I) RT-qPCR data demonstrating that SIS3 treatment effectively negates the SCPPPQ1-driven upregulation of Runx2 and ALP mRNA. (J) Western blot verification showing the reduction of Runx2, ALP, and phosphorylated Smad3 (P-Smad3) proteins following inhibitor treatment. GAPDH served as the loading control, and P-Smad3 protein levels were normalized to GAPDH. Data represent the mean $\pm$ SD from three independent biological replicates. “ns” denotes not significant, * p < 0.05, ** p < 0.01. Runx2, Runt-related transcription factor 2; SIS3, specific inhibitor of Smad3.

we introduce a new layer to this narrative. We provide evidence that SCPPPQ1 also plays a critical role in intracellular signaling, driving ameloblast differentiation and mineralization by modulating this exact TGF- β 1–Runx2/ALP pathway. This positions SCPPPQ1 as a bifunctional protein operating at the cell–matrix interface—seamlessly blending its adhesion duties with active signal transduction. This dual function perfectly makes sense for late-stage amelogenesis. During this phase, ameloblasts have to hold on tight to the enamel surface while simultaneously running highly complex ion transport and matrix remodeling programs to fully mineralize the enamel. A logical hypothesis is that SCPPPQ1 acts as a vital signaling node. It likely “reads” the extracellular environment, such as matrix composition, cell attachment, or mechanical stress, and translates those cues into intracellular signals, fine-tuning the downstream Smad3-dependent transcriptional outputs (Fig. 9). Although we cannot yet confirm if SCPPPQ1 functions directly as a mechanosensor, linking an adhesion complex

to TGF- β pathway activity is highly biologically plausible. This concept bridges SCPPPQ1’s well-known extracellular location with its newly discovered intracellular signaling role.

Finally, this SCPPPQ1-centered regulatory axis may carry significant translational potential. Unpacking the precise mechanics of the SCPPPQ1–TGF- β 1–Runx2/ALP module could pave the way for new enamel repair strategies, offering hope for patients suffering from hypomineralized enamel disorders. While true enamel regeneration remains notoriously difficult since ameloblasts are lost after tooth eruption, identifying these molecular “switches” provides us with actionable targets for future biomimetic or cell-based therapies. In this light, SCPPPQ1 stands out as a prime candidate for therapeutic intervention. By either boosting its native activity or targeting its downstream signaling cascade, we might finally be able to improve clinical mineral deposition outcomes.

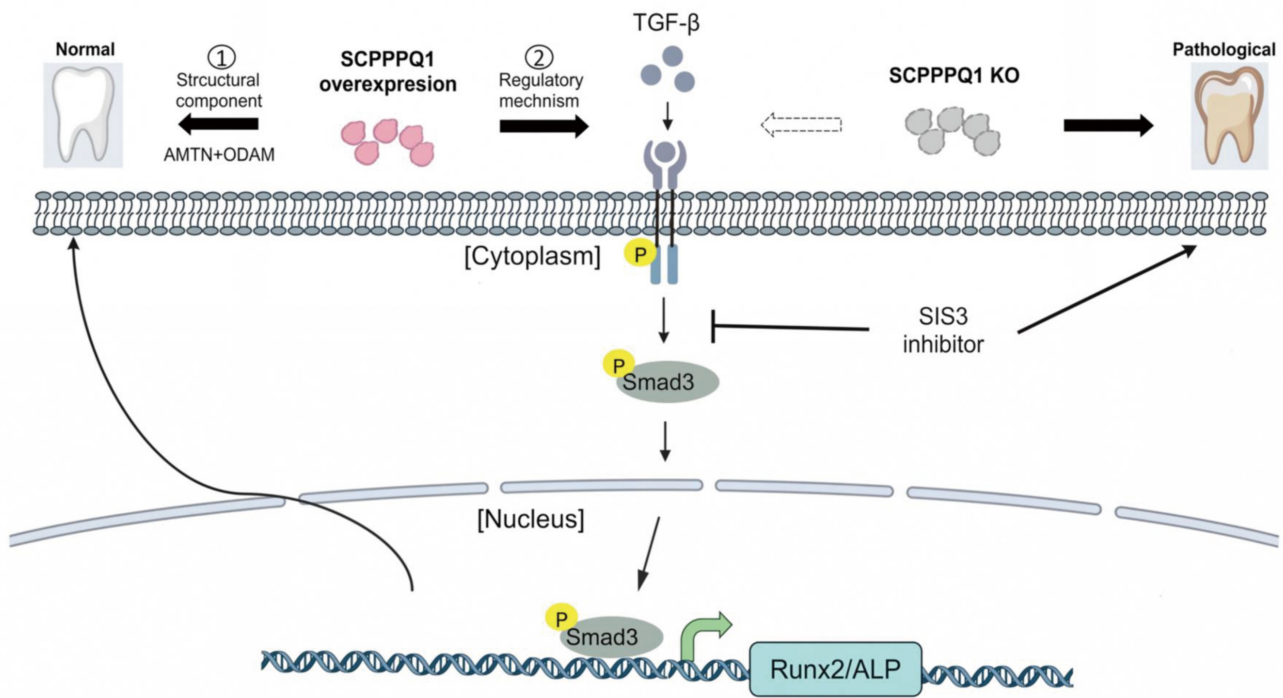


Fig. 9. Schematic model illustrating the dual role of SCPPPQ1 in tooth development. Under normal physiological conditions, SCPPPQ1 serves as a structural matrix component alongside AMTN and ODAM (①). Beyond this structural function, it also acts as a critical molecular regulator (②) that may regulate TGF- β receptor signaling at the cell membrane. This receptor activation drives the cytoplasmic phosphorylation of Smad3 (P-Smad3), which subsequently translocates into the nucleus. There, it upregulates downstream osteogenic targets, including Runx2 and ALP, thereby ensuring proper enamel mineralization and tooth structure. In contrast, the genetic deletion of SCPPPQ1 (KO) breaks this signaling cascade, leading to the defective enamel phenotype. Similarly, blocking Smad3 activation with the SIS3 inhibitor mimics this pathology, which supports the involvement of the TGF- β 1/Smad3 signaling pathway in normal tooth development. AMTN, Amelotin; ODAM, Odontogenic Ameloblast-Associated Protein.

5. Conclusion

In conclusion, this study illuminates the critical regulatory role of SCPPPQ1 in enamel mineralization. Using an *SCPPPQ1* KO mouse model, we first confirmed that lacking this protein causes severe enamel hypomineralization and structural defects *in vivo*. Shifting to our *in vitro* ALC model, we found that overexpressing SCPPPQ1 enhances the cells' mineralization capacity, a shift driven by the upregulation of key osteogenic markers like Runx2 and ALP. Mechanistically, we pinpointed the TGF- β 1 signaling pathway as the vital engine behind this process. Blocking this pathway with the inhibitor SIS3 abolished the promineralization effects and gene expression gains induced by SCPPPQ1. Ultimately, these findings map out a novel regulatory axis where SCPPPQ1 drives ameloblast mineralization via the TGF- β 1-mediated activation of Runx2 and ALP. This provides a perspective on the molecular blueprints governing amelogenesis.

6. Limitations and Future Directions

A primary limitation of our study is the current lack of *in vivo* pathway validation. We acknowledge that repeated sectioning has exhausted all original tissue blocks,

rendering re-staining and re-imaging for this study impossible. Because of this, our mechanistic findings—specifically that SCPPPQ1 regulates enamel mineralization through the TGF- β 1 pathway, remain confined to our *in vitro* cell models. Without the *in vivo* confirmation, we cannot definitively prove the physiological stability of this specific signaling loop in living tissues, which naturally limits its immediate jump to clinical translation. Moving forward, our research will branch into two main directions. First, we plan to design rigorous *in vivo* rescue experiments. This will allow us to validate the specificity of the SCPPPQ1/TGF- β 1 axis and further validate its upstream-downstream relationship in a living organism. Second, we aim to find out exactly how SCPPPQ1 pulls the levers of this pathway—specifically, whether it interacts directly with TGF- β 1 or binds to its receptors. Answering these questions will fill in the remaining puzzle pieces regarding the complex regulatory networks that govern enamel formation.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Author Contributions

WG: Conceptualization, Methodology, Investigation, Formal analysis, Writing—Original Draft. ZX: Visualization. YW: Carried out biological interpretation of the experimental data and revised the conclusions, Writing—Review & Editing. HM: Visualization. QC: Visualization. YT: Resources, Visualization. GZ: Data curation. LX: Conceptualization, Supervision, Writing—Review & Editing. YG: Conceptualization, Supervision, Writing—Review & Editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All procedures complied with the ARRIVE guidelines and the national standard GB/T 35892-2018 for laboratory animal welfare. The protocol was approved by the Animal Ethics Committee of Binzhou Medical University (Approval No. 2025-L029).

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/FBL53495>.

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