HHS Public Access

Author manuscript

Dev Dyn. Author manuscript; available in PMC 2024 October 01.

Published in final edited form as:

Dev Dyn. 2023 October ; 252(10): 1292–1302. doi:10.1002/dvdy.598.

Enam expression is regulated by Msx2

Intan Ruspita[1,2,#], **Pragnya Das**[1,3,#], **Keiko Miyoshi**[4], **Takafumi Noma**[5], **Malcolm L. Snead**[6], **Marianna Bei**[7,8,9,*]

[1]Center for Regenerative and Developmental Biology, The Forsyth Institute, Cambridge, MA, USA

[2]Universitas Gadjah Mada, Yogyakarta, Indonesia

[3]Cooper University Hospital, Camden, NJ, USA

[4]Department of Molecular Biology, Institute of Biomedical Sciences, Tokushima University, Tokushima, Japan.

[5]Faculty of Human Life Studies, Hiroshima Jogakuin University, Hiroshima, Japan

[6]Center for Craniofacial Molecular Biology, Herman Ostrow School of Dentistry of USC, University of Southern California, LA, CA

[7]Center for Engineering in Medicine and Surgery, Department of Surgery, Massachusetts General Hospital, Boston MA, USA

[8]Department of Surgery, Harvard Medical School, Boston MA, USA

[9]Shriners Hospital for Children, Boston, MA

Abstract

Background: The precise formation of mineralized dental tissues such as enamel and/or dentin require tight transcriptional control of the secretion of matrix proteins. Here we have investigated the transcriptional regulation of the second most prominent enamel matrix protein, enamelin, and its regulation through the major odontogenic transcription factor, MSX2.

Results: Using in vitro and in vivo approaches, we identified that (i) *Enam* expression is reduced in the *Msx2* mouse mutant pre-secretory and secretory ameloblasts, (ii) *Enam* is an early response gene whose expression is under the control of Msx2, (iii) Msx2 binds to *Enam* promoter *in vitro,* suggesting that *enam* is a direct target for *Msx2* and that (iv) Msx2 alone represses *Enam* gene expression.

[*]Correspondence: **author:** Dr. Marianna Bei, Department of Surgery, Massachusetts General Hospital & Harvard Medical School, Shriners Hospital for Children-Boston, mbei@mgh.harvard.edu.

[#]:equal contribution

Author Contributions: MB contributed to the concept and design of the study. IR and MB contributed to the acquisition of data. IR, PD, and MB prepared the figures. IR, PD, and MB contributed to the data analysis and interpretation. IR, PD and MB wrote the manuscript. IR, PD, MLS, TN, and MB contributed to the editing and the critical revision for intellectual content. All authors have approved the final version of the submitted manuscript.

Ethics Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Massachusetts General Hospital, Boston, MA (2013N000213, Date: 12/23/2019).

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Conclusions: Collectively, these results illustrate that *Enam* gene expression is controlled by Msx2 in a spatio-temporal manner. They also suggest that Msx2 may interact with other transcription factors to control spatial and temporal expression of Enam and hence amelogenesis and enamel biomineralization.

Keywords

Msx2 ; *enamelin* ; transcriptional regulation

INTRODUCTION

Amelogenesis is the process of enamel formation. It takes place in three, well-defined stages known as the secretory, transition and maturation phases and the cell mediating this process is the ameloblast. The ameloblast starts its differentiation process as an epithelial proliferative cell, separated from adjacent mesenchyme by a basement membrane. The initial differentiation, orientation of ameloblasts and their coordinated function are critical to amelogenesis and involves the secretion of a proteinaceous matrix in which immature enamel hydroxyapatite (HA) crystallites are deposited. The matrix is then degraded and replaced, almost entirely, with HA mineral. The functional ameloblasts express numerous proteins, including enzymes, signaling molecules, cell-cell adhesion molecules, transcription factors and several secreted proteins, such as *ameloblastin*, *amelogenin*, *enamelin*, *tuftelin*, *DSPP*, *apin*, *amelotin* [1,2,3]. Of all secreted proteins, amelogenin, enamelin and ameloblastin are the major secretory products of ameloblasts that contribute to enamel formation [4,5]. Amelogenin is the most abundant one, while ameloblastin and enamelin are less abundant and degrade rapidly during enamel formation [6].

Enam is uniquely expressed by the ameloblasts and is expressed during the secretory, transition and early maturation stages of ameloblast life cycle [7,8]. *In silico* analysis of mammalian and non-mammalian tetrapods indicates that the (i) *ENAM* gene has originated very early in vertebrate evolution with 25 amino acids of its sequence to be conserved for 350 million years of tetrapod evolution, and (ii) its regulation during evolution is critical for attributing correct *ENAM* functions to different species [9,10].

Many genes when mutated have been demonstrated to result in non-syndromic and syndromic AI. In humans, mutations in *ENAM* gene are associated with non-syndromic amelogenesis imperfecta (AI), a heterogeneous group of genetic conditions characterized by defects in the formation of enamel and are found in non-syndromic conditions that affect only the enamel formation of teeth, or are part of congenital disorders, such as ectodermal dysplasias affecting more than one ectodermal organ. The first mutation identified in *ENAM* gene resulted in a dominant-negative effect of aberrant splicing causing an autosomal dominant AI with a severe, smooth hypoplastic phenotype (MIM #104500) [11]. Another milder, local hypoplastic phenotype (MIM #204650) is caused by missense mutations in *ENAM* [12], while other autosomal recessive inheritance mutations have also been documented [13, 14, 15, 16]; http://dna2.leeds.ac.uk/LOVD/). On the other hand, syndromic AI are part of congenital disorders and the genes responsible for these disorders control the development and/or maintenance of many other organs.

Animal and human data indicate that transcription factors are critical for the formation of several organs and structures including the tooth enamel [2]. Among them is the transcription factor Msx2. Mice lacking the homeobox gene *Msx2* exhibit defects in several ectodermal organs including the tooth and the process of amelogenesis. In the *Msx2* knock out (KO) mice the ameloblasts secrete sparse amounts of enamel matrix [2, 17, 18, 19, 20]. Detailed morphometric analysis revealed that the amount of enamel deposited by the mutant ameloblasts is < 2% the amount of enamel deposited by the wild type ameloblasts [2, 17, 19]. These results show that depletion of *Msx2* function causes abnormalities in amelogenesis, by controlling the ameloblast differentiation process and enamel production. Several reports suggest a possible role of *Msx2* in regulating enamel formation through the control of downstream genes such as, *laminin 5 alpha 3*, *follistatin*, *amelogenin, DSPP, cytokeratin 5*, MMP20, KLK4, all of which are equally important for enamel formation [2, 17–27]. The role of transcription factors such as *Msx2* in directly regulating any of the enamel proteins, however, is not extensively studied except for *amelogenin* [8, 18, 21, 28, 29, 30].

In the present study, we show that (i) *Enam* expression is reduced in the *Msx2* mouse mutant pre-secretoty and secretory stage ameloblasts, (ii) *Enam* is an early response gene whose expression is under the control of Msx2, (iii) Msx2 binds to *Enam* promoter *in vitro*, (iv) Msx2 alone represses *Enam* gene expression. These results suggest that *Enam* gene expression is partially under the control of Msx2 regulation in a spatio-temporal manner. They also suggest that Msx2 may interact with other transcription factors and that these interactions could relieve repression allowing thus *Enam* to be expressed, or *Enam* is under the control of multiple transcription factors that coordinately control its spatial and temporal expression.

RESULTS

Msx2 is necessary for Enam gene expression during late tooth development

To determine whether *Msx2* is required for *Enam* regulation and whether this requirement is associated with the defect in amelogenesis, *in situ* hybridization was performed in wild type and *Msx2* deficient mouse molar and incisor tooth germs at postnatal day 1 (P1), postnatal day 3 (P3), postnatal day 6 (P6) and postnatal day 9 (P9) (Fig. 1A–P). *Msx2* is expressed by pre-secretory ameloblasts (P1), secretory ameloblasts (P3-P6) and is not expressed by (P9) when ameloblasts are at the maturation stage [2,3, 17, 18, 26, 31, 32]. A dramatic reduction of *Enam* expression is observed in *Msx2* deficient molars and incisors compared to wild type ones at P1 (Fig. 1A–D), P3 (Fig. 1E–H) and P6 (Fig. 1I–L) when ameloblasts are at their pre-secretory and secretory stage. This result indicates that *Enam* requires *Msx2* for its expression in the pre-secretory and secretory stage ameloblasts. At P9, *Msx2* is not expressed by mature ameloblasts, while *Enam* is restricted to the lower buccal and lingual sides of the crown around the cervical area (Fig. 1M). The expression of *Enam* showed no considerable difference between wild-type and *Msx2*-deficient tooth germs at this stage, considering the qualitative nature of the ISH (Fig. 1O). In the *Msx2* deficient incisors even a slight increase in *Enam* expression is observed compared to wild type ones, at P9 (Fig. 1N, P).

To test whether *Msx2* regulates the expression of *Enam*, in a quantitative manner, we performed real time quantitative PCR. Total RNA from P1, P3, P6 and P9 first molar tooth germs was extracted, reverse transcribed and qPCR was performed. We show that the expression of *Enam* is downregulated in the *Msx2* deficient P1, P3 and P6 tooth germs, but not at P9, further confirming our in vivo results (Fig. 1, **lower panel**). The higher expression of *Enam* in the *Msx2* deficient teeth compared to wild type ones at P9 qPCR is the result of the cumulative expression of *Enam* from the buccal and lingual sides of the crown of the molars.

We also performed loss-of and gain-of function studies in LS8 cells because this cell line is an ideal model to test gene regulation during tooth development [19, 27]. Specifically, we tested the effects of acute knockdown of *Msx2* in LS8 ameloblast-derived cells and compared to what happens in development where Msx2 is permanently absent in the *Msx2* deficient mice (Fig. 2A–D). For the knock down experiment, we used siRNA technology in LS8 cells (Fig. 2A, B). After 48 and 72 hrs transfection the cells were subjected to real time quantitative PCR. We found that upon transient silencing of *Msx2*, *Enam* was downregulated, further suggesting that *Enam* requires Msx2 for its expression (Fig. 2A, B). In addition, we used lentiviral shRNA mediated approach to assess the effects of permantly silencing *Msx2* gene. Specifically, the LS8 cells were infected with mouse Msx2shRNA lentiviral transduction particles. qPCR shows that Msx2shRNA lentiviral transduction particles effectively reduce *Enam* gene expression in LS8 cells compared to control shRNA treated cells (Fig. 2C, D). For the gain-of function assays, induction of *Msx2* was achieved through transient transfection of *Msx2* expression plasmid into LS8 cell lines. Overexpression *Msx2* in both cell lines results in significant increase of *Enam* expression (Fig. 3A).

The expression of enam is modulated early in response to Msx2

To determine the kinetics of *enam* gene expression, we performed a time dependent assay to ascertain whether its expression is modulated early in response to *Msx2* upregulation (Fig. 3B). LS8 cells were transfected with *Msx2* over-expression plasmid for several time points, 4, 8, 16, 24 and 48 hours. Total RNA was isolated from the cells and subjected to qPCR analysis. *Enam* could be detected as early as 4h after transfection by real time qPCR (Fig. 3B). *Enam* levels increased immediately within 4 hours of Msx*2* expression and reached a maximum peak at 16 hours post transfection, following which there was a gradual decline around 24h and subsequently in 48h (Fig. 3B). We did not see any significant response earlier than 4 hours; thus, these results indicate that *Enam* is secondary early response gene to *Msx2* (response after 4 hours of *Msx2* overexpression).

Msx2 directly binds to Msx2 recognition sites on the Enam promoter

The loss-of and gain-of-function studies along with the *in vivo* experiments using the *Msx2* deficient mice show that Msx2 is required for the expression of *Enam* in the pre-secretory and secretory ameloblasts, during amelogenesis. Computational sequence analysis of the nucleotides in the proximal 5.2kb of the murine *Enam* promoter region revealed the presence of 3 putative homeodomain binding sites upstream from the transcription initiation site in the mouse (Fig. 4A). To determine whether Msx2 binds to any of these sites and

therefore, directly regulates *Enam*, chromatin immuno-precipitation was performed with exogenously expressed Msx2-FLAG in LS8 cells and P3 wild type mouse M1 molar tooth germs. Immunoprecipitated chromatin fragments (IP samples) and non-immunoprecipitated samples (1% input) were subjected to PCR analysis using specific primers spanning the three binding sites. PCR amplifications showed that Msx2 binds directly to all three putative sites carrying the conserved motif (TAAT) in the endogenous promoter of the mouse *Enam* gene, −500TAATta, −2000TAATtta (weak, not shown) and −2900TAAttc (Fig. 4B, C). This result demonstrates that Msx2 binds directly to the proximal *Enam* promoter *in vitro* and *in vivo*.

To determine if *Enam* promoter is repressed or activated by Msx2, LS8 cells were co-transfected with *Enam*-luciferase reporter plasmid and Msx2 overexpression plasmid (Fig. 4D) and luciferase activity measured. We found that transfection of Msx2 with 3 putative binding sites resulted in a repression of the *Enam*-luciferase reporter plasmids. This shows that Msx2 acts as a repressor, suppressing but not alleviating the expression of *Enam*, acting as a dosage regulator.

In sum, we show that (i) *Enam* expression is reduced in the *Msx2* mouse mutant pre-secretory and secretory ameloblasts, (ii) *Enam* is an early response gene whose expression is under the control of Msx2, (iii) Msx2 binds to *Enam* promoter *in vitro,* suggesting that *Enam* is a direct target for *Msx2* and that (iv) Msx2 alone represses *Enam* gene expression. Collectively, these results show that *Enam* gene expression is partially under the control of Msx2 regulation in a spatio-temporal manner through a remote enhancer. They also suggest that Msx2 may interact with other transcription factors that coordinately control its spatial and temporal expression.

DISCUSSION

Msx2 is required for Enam expression

Ameloblast differentiation program is impaired in *Msx2*$^{-/-}$ mice leading to enamel dysplasia [20]. *Msx2* is expressed by preameloblasts, early secretory, secretory ameloblasts and ceases to be expressed by maturation stage ameloblasts [17–19, 26, 31, 32]. On the other hand, *Enam* expression is initiated early during the preameloblast stage and continues through the secretory and early maturation stages of ameloblast life cycle[1]. Our *in vivo* data indicate that at P1, P3 and P6 when *Msx2* and *Enam* are co-expresssed, *Enam* requires Msx2 for its expression. At P9, when the ameloblasts are at the maturation stage and *Msx2* is no longer expressed, the *Msx2* mutant ameloblasts continue to express *Enam* (Fig. 1). In addition to our *in vivo* data, our gain of function, loss of function and time dependent assay further confirm that *Enam* requires Msx2 for its expression. Our results are also consistent with RT-qPCR analysis that revealed reduced expression of both *amelogenin* and *Enam* in *Msx2*$^{-/-}$ mouse dental epithelium [18]. Moreover, our characterization of *Enam* promoter for Msx2 binding sites revealed three putative Msx2 binding sites and our ChIP experiments provided evidence that Msx2 binds to *Enam* promoter, suggesting that *Enam* is a target for Msx2. Based on the above, we could potentially draw the conclusion that Msx2 may promote *Enam* expression directly acting, as an activator of *Enam* expression. Our luciferase

experiments, on the other hand, indicate that Msx2 alone represses *Enam* expression. How results apparently contradictory may find an explanation?

Msx2 partially regulates Enam potentially in concert with other repressors and activators.

Msx2 transcription factor is known to act as, both, a repressor [33, 34] and activator [35] and, like most transcription factors, Msx2 does not act alone but rather in concert with other transcription factors to regulate the final dosage and the onset of expression of downstream genes [2, 21, 25]. In that context, *Enam's* expression during the presecretory and secretory stages may be partially regulated by Msx2, in concert with additional repressors and/or activators. These transcription factors may interact with Msx2 physically and/or *in vivo* via a protein-protein interaction mechanism to control *Enam* expression level, like what it has been shown for another secreted protein, the amelogenin. Msx2 is shown to interact with C/EBPα to repress the promoter activity of amelogenin-promoter reporter constructs independent of its intrinsic DNA binding activity. In transient co-transfection assays, Msx2 and C/EBPα antagonize each other in regulating the expression of the mouse *amelogenin* gene [21].

For *Enam* we know that co-transfection analysis and mutation assays revealed two conserved LEF1 responsive elements located at −1002 and −597bp upstream of the *Enam* translation initiation site that could augment transcriptional activity of the *Enam*, suggesting that the beta-Catenin/LEF1 is a key transcriptional complex regulating transcriptional activity of the *Enam* [36]. Runt-related transcription factor 2 (Runx2) is also involved in amelogenesis. In the *Runx2* conditional knockout (cKO) mouse, qRT-PCR analysis revealed that the expression of *Enam* was increased suggesting that Runx2 may act as a repressor of *Enam* gene [37, 38]. These results were further confirmed by *in vitro* studies showing that *Enam* expression levels were downregulated in *Runx2* over-expressing cells [39]. Athanassiou-Papafthymiou and colleagues also showed that *Enam* expression levels were subject of Dlx3 transcription factor regulation. *Enam* expression was up-regulated in *Dlx3* over-expressing cells [39, 40], whereas knockdown of Dlx3 down-regulated its expression [41]. More importantly, chIP and luciferase assays have shown that DLX3 transactivated *Enam* [41], most probaby through a potential *cis*-regulatory element for *Enam* located 5.2kb upstream of the enamelin translation inititation site. This *cis*-regulatory element found to be sufficient to drive endogenous *Enam* in ameloblast cells using transgenic mice [8, 40]. This indicated that DLX3 participates in the tissue-specific expression of *Enam* in ameloblasts. Interestingly, when we analyzed this 5.2kb region using MatInspector software, we found 3 putative *Msx2* binding sites between −3900~−500bp region (Fig.4A), that Msx2 binds strongly and directly to −500bp and −2900bp regions (Fig. 4B) and that Msx2 alone represses *Enam* (Fig. 4C).

Considering the forementioned studies and in the context of our findings, we propose that except for Msx2, other transcription factors, such as Lef1, Runx2 or Dlx3, may keep on regulating and fine-tuning *Enam's* expression level during the presecretory and secretory stages [36–42]. It is also quite possible that *Msx2* may recruit unknown or uncharacterized factors, that may work in concert as transcriptional repressors or activators in a time and context dependent manner to regulate onset and right dose of *Enam* gene expression. Indeed, in addition to *Msx2*, we have identified multiple binding sites for several other transcription

factors like *Sox9*, *Dlx1*, *Isl1*, *Lhx6*, *Pax6*, *Nfy*, and *Sp3* in proximity with the *Msx2* binding sites in the *Enam's* regulatory region (data not shown). This suggests that besides *Msx2, Dlx3* and *Runx2* binding sites, the regulatory regions of the *Enam* promoter may also contain several binding sites for other transcription factors that could act as negative or positive regulators of its overall expression.

In sum, it is obvious that Msx2 is playing an important role in regulating amelogenesis but not alone, but rather in concert with other transcription factors. Although additional studies will help to better understand the relationship between Enam and Msx2, it seems that the right dose of enamelin is essential, and it is critical for amelogenesis in general as it was further demonstrated in transgenic mouse lines over-expressing *enamelin*. Hu and colleagues have shown that by introducing enamelin transgene at various expression levels into the *Enam*$^{-/-}$ background did not fully recover enamel formation while a medium expresser in the *Enam*$^{+/-}$ background did"[43]. "Thus, too much or too little *enamelin* is essential for ameloblast integrity and enamel formation" [18, 43]. If *Enam* acts in a dose-dependent manner, our data indicates that its biological function is dictated by a network of transcription factors including Msx2 that fine-tunes its optimum dosage and onset of full expression in a spatial and temporal manner.

EXPERIMENTAL PROCEDURES

Mice and genotyping

All animal studies and experimental procedures were conducted in accordance to the guidelines for the care and use of laboratory animals by the Massahusetts General Hospital, Boston, MA and the Forsyth Institute, Cambridge, MA. Postnatal pups (P1, P3, P6 and P9) were collected from matings of *Msx2* heterozygous animals maintained in BALB/c background. Genotyping was performed as previously described [17, 19]. Age matched wildtype pups and/or embryos served as the appropriate controls.

In situ hybridization (ISH)

Postnatal animals (P1, P3, P6, P9) were collected and heads decapitated for making coronal and sagittal sections. P1 and P3 samples were immediately fixed in freshly made 4% paraformadehyde while P6 and P9 samples were decalcified in 12.5% EDTA+2.5% PFA/PBS-DEPC at 4°C for 1 week. All samples were then dehydrated through graded ethanol series, embedded in paraffin, sectioned at 8μm and processed for ISH, as previously described [17, 19]. Sense (5'CCAGACTTCCTGCCTCAAAG 3'*)* and antisense primers (5'AGGACTTTCAGTGGGTGTGG 3'*)* were used to synthesize the *enamelin* probe in a PCR reaction. T7 primer sequence sites were added to the antisense sequence to generate the antisense probe by PCR method. The PCR products were gel purified (Qiagen Inc, Valencia, CA), labeled with DIG-UTP (Roche Biochemica, Mannhein, Germany) and used directly for hybridization. The sense probe was used as a negative control.

Cell culture

Two different dental epithelial cell lines were used in the present study – the rat dental epithelial cell line (G5) and the mouse dental epithelial cell line (LS8), shown [19]. Both cell

lines were maintained in high-glucose Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA), containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37 °C in 5% CO2 humidified atmosphere following the standard protocols [27].

Gain-of-Function and Loss-of-Function Experiments

For overexpression of *Msx2*, LS8 cells were transfected with pCMVtag2-Flag-*Msx2*, and then cultured for 48h-72h following which total RNA was isolated from the cells using Trizol (Qiagen, MD, USA). An empty vector (pCMVtag2) served as a negative control for gain-of-function studies. For loss of function of *Msx2*, commercially available small interfering RNA for *Msx2* (*Msx2*-siRNA) was used (Santa Cruz, CA, USA). We used the following oligonucliotides, sense sequence 5'CAGCUCUCUGAACCUUAC 3' (sc-43947). As negative control we used a scramble sequence that will not lead to the specific degradation of any known cellular mRNA: sense scramble control 5'UUCUCCGAACGUGUCACG 3' (sc-37007). Overexpression and gene knockdown studies were performed following the protocol as described [19].

Time dependent assay

LS8 cells were transfected with pCMVtag2-Flag-*Msx2* (Invitrogen, USA) and then cultured for up to 48h. The cells were harvested at different time points (4h, 8h, 16h, 24h and 48h) for RNA isolation and subjected to real time qPCR analysis to check for time-dependent expression of *Msx2* and *enamelin*. These experiments have not been performed in the presence of the protein synthesis inhibitor, cycloheximide and thus, we do not know whether the secondary response of *Enam* gene require *de novo* protein synthesis for transcription.

Real-time quantitative PCR

Total RNA from LS8 cells and mice P1, P3, P6 and P9 molar tooth germs (M1) was extracted with Trizol according to manufacturer's instructions and reverse transcription was performed using qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). Quantitative PCR was carried out in LightCycler and LightCycler-Faststart DNA Master SYBR Green I (Roche Diagnostics, Switzerland). The expression level of each sample was normalized to *Gapdh* (glyceraldehyde-3phosphate dehydrogenase) mRNA expression. The primers used are as follows:

Msx2: **F** 5'AGACATATGAGCCCCACCAC 3'/**R** 5'CAAGGCTAGAAGCTGGGATG 3'

Enamelin: **F** 5' TCCAGGAAACCCAACTTACG 3'/**R** 5'TTTCTTCCGAAATGGACTGG 3'

GAPDH: **F**5'GCAAAGTGGAGATTGTTGCCAT3'/
R5'CCTTGACTGTGCCGTTGAATTT3'

Reporter Construct

The 5'-flanking region of the mouse *enamelin* gene was generated by PCR using AccuStart taq DNA polymerase HiFi (Quanta, MD, USA), according to the manufacturer's instructions. Three different constructs were made using the following PCR primers:

pGL3-4578: F 5'CCCGGGCTCGAGATCTGTAACTACTACCTTTGAGGGC 3'

R 5'CCGGAATGCCAAGCTTAGAGAGAGCCAAGGAGCAAGA 3'

pGL3-500: F 5'CCCGGGCTCGAGATCTCCTAACAACGAAGCTACATCTG 3'

R 5'CCGGAATGCCAAGCTTTTATTACCATCAACCATACCCTTA 3'

pGL3-2000: F 5'CCCGGGCTCGAGATCTTATGTCAATGTAAACAGTGTTATGC 3'

pGL3-2900: F 5' CCCGGGCTCGAGATCTGGTCCCAGACTAAGAAGGCT 3'

The reverse primer was common for **the pGL3-500**, **pGL3-2000** and **pGL3-2900** constructs.

The amplified products were extracted and purified with NucleoSpin Extract II and cloned into **pGL3-Basic** vector (Promega, Madison, WI) using Fusion HD Cloning kit (Clontech, Mountain View, CA, USA). All constructs were confirmed by DNA sequencing.

Luciferase Assay

The *enamelin*-luciferase reporter plasmids were constructed using Enamelin 5'flanking regions encompassing the putative Msx2 binding sites (as predicted from UCSC Genome browser and MATINSPECTOR), and cloned into pGL3 basic luciferase vector (Promega, USA). The Reporter vector was transfected into LS8 cells together with pCMV-FLAG-*Msx2* or vector only (negative control) and phRLTK (as normalizing internal control) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions for 48h, following which the cells were harvested, the luciferase activity recorded using Promega kit, after normalizing with firefly/renilla luciferase activity. The data was obtained from three independent experiments, and each experiment was done in triplicates.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (chIP) was performed using the EZ-Magna chip kit (Millipore, Billerica, USA) according to the manufacturer's instructions. Forty-eight hours after transfection with pCMV-FLAG-*Msx2* expression plasmid, LS8 cells and P3 wild type mouse M1 molar tooth germs were fixed and crosslinked with 1% (v/v) formaldehyde at 37°C for 10 min. Crosslinking was stopped by adding glycine to a final concentration of 125mM, followed by washing with cold PBS. After sonication chromatin was incubated with magnetic beads conjugated to either 1μg of monoclonal anti-Flag antibody (F3165, Sigma) or 1μg of normal mouse IgG (EZ-Magna chip kit) or anti-Msx2. Immunoprecipitated chromatin was reverse crosslinked and washed before DNA extraction. Finally, the

immunoprecipitated DNA and the corresponding non-immunoprecipitated DNA (input) was subjected to PCR using the following enamelin primers:

F1: 5’ TTGGCCAGCTCCTCTAAAAG 3’/ **R1** 5’ CACTGGCCACCATCAAAAG 3’

F2: 5’ TATGCTCACTACTCAATTAC 3’/ **R2** 5’ CGTAGTTCCAAAGTTTAGTG 3’

F3: 5’ GGGAGGCAAGTGGATATTT 3’/ **R3** 5’ CGGACGTGACTTTTCTCCAT 3’

Control-F: 5’TCCATTCCCTGGTATCCTGA 3’/ **R** 5’ CCAAAATTCACCCATCCATT 3’

In silico analysis of promoter binding sites

UCSC MatInspector software was used to predict the putative promoter binding regions for Msx2. Primers were designed from these predicted regions using Primer 3 database for chIP followed by PCR amplification.

Imaging

The imaging for ISH was done using Olympus microscope.

Statistics

Each cell culture experiment was replicated 3 times. For ISH, a minimum of 3–4 mice pups were used. Statistics was done using one-way ANOVA or students t-tailed test, wherever applicable using GraphPad prism (version 7, CA). P value of <0.05 was considered statistically significant.

Funding:

The study was supported by funds from NIH [grant, R21DE028091] and the MGH Executive Committee Of Research to M.B. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data Availability Statement:

All datasets generated for this study are included in the article.

REFERENCES

1. Hu JC, Hu Y, Smith CE, et al. Enamel defects and ameloblast-specific expression in Enam knock-out/lacz knock-in mice. J Biol Chem 2008;283(16):10858–10871. doi:10.1074/jbc.M710565200 [PubMed: 18252720]
2. Bei M Molecular genetics of ameloblast cell lineage. J Exp Zool B Mol Dev Evol 2009;312B(5):437–444. doi:10.1002/jez.b.21261 [PubMed: 19090561]
3. Bei M Molecular genetics of tooth development. Curr Opin Genet Dev 2009;19(5):504–510. doi:10.1016/j.gde.2009.09.002 [PubMed: 19875280]
4. Fincham AG, Moradian-Oldak J, Simmer JP. The structural biology of the developing dental enamel matrix. J Struct Biol 1999;126(3):270–299. doi:10.1006/jsbi.1999.4130 [PubMed: 10441532]
5. Siddiqui S, Al-Jawad M. Enamelin Directs Crystallite Organization at the Enamel-Dentine Junction. J Dent Res 2016;95(5):580–587. doi:10.1177/0022034516632745 [PubMed: 26912218]

6. Smith CE, Wazen R, Hu Y, et al. Consequences for enamel development and mineralization resulting from loss of function of ameloblastin or enamelin. Eur J Oral Sci 2009;117(5):485–497. doi:10.1111/j.1600-0722.2009.00666.x [PubMed: 19758243]
7. Daubert DM, Kelley JL, Udod YG, et al. Human enamel thickness and ENAM polymorphism. Int J Oral Sci 2016;8(2):93–97. doi:10.1038/ijos.2016.1 [PubMed: 27357321]
8. Papagerakis P, Hu Y, Ye L, Feng JQ, Simmer JP, Hu JC. Identifying promoter elements necessary for enamelin tissue-specific expression. Cells Tissues Organs 2009;189(1–4):98–104. doi:10.1159/000151429 [PubMed: 18703866]
9. Al-Hashimi N, Lafont AG, Delgado S, Kawasaki K, Sire JY. The enamelin genes in lizard, crocodile, and frog and the pseudogene in the chicken provide new insights on enamelin evolution in tetrapods. Mol Biol Evol 2010;27(9):2078–2094. doi:10.1093/molbev/msq098 [PubMed: 20403965]
10. Kawasaki K, Keating JN, Nakatomi M, et al. Coevolution of enamel, ganoin, enameloid, and their matrix SCPP genes in osteichthyans. iScience 2021;24(1):102023. Published 2021 Jan 1. doi:10.1016/j.isci.2020.102023 [PubMed: 33506188]
11. Rajpar MH, Harley K, Laing C, Davies RM, Dixon MJ. Mutation of the gene encoding the enamel-specific protein, enamelin, causes autosomal-dominant amelogenesis imperfecta. Hum Mol Genet 2001;10(16):1673–1677. doi:10.1093/hmg/10.16.1673 [PubMed: 11487571]
12. Mårdh CK, Bäckman B, Holmgren G, Hu JC, Simmer JP, Forsman-Semb K. A nonsense mutation in the enamelin gene causes local hypoplastic autosomal dominant amelogenesis imperfecta (AIH2). Hum Mol Genet 2002;11(9):1069–1074. doi:10.1093/hmg/11.9.1069 [PubMed: 11978766]
13. Hart TC, Hart PS, Gorry MC, et al. Novel ENAM mutation responsible for autosomal recessive amelogenesis imperfecta and localised enamel defects. J Med Genet 2003;40(12):900–906. doi:10.1136/jmg.40.12.900 [PubMed: 14684688]
14. Kim JW, Simmer JP, Lin BP, Seymen F, Bartlett JD, Hu JC. Mutational analysis of candidate genes in 24 amelogenesis imperfecta families. Eur J Oral Sci 2006;114 Suppl 1:3–379. doi:10.1111/j.1600-0722.2006.00278.x [PubMed: 16674655]
15. Lindemeyer RG, Gibson CW, Wright TJ. Amelogenesis imperfecta due to a mutation of the enamelin gene: clinical case with genotype-phenotype correlations. Pediatr Dent 2010;32(1):56–60. [PubMed: 20298654]
16. Ozdemir D, Hart PS, Firatli E, Aren G, Ryu OH, Hart TC. Phenotype of ENAM mutations is dosage-dependent. J Dent Res 2005;84(11):1036–1041. doi:10.1177/154405910508401113 [PubMed: 16246937]
17. Bei M, Stowell S, Maas R. Msx2 controls ameloblast terminal differentiation. Dev Dyn 2004;231(4):758–765. doi:10.1002/dvdy.20182 [PubMed: 15499554]
18. Molla M, Descroix V, Aïoub M, et al. Enamel protein regulation and dental and periodontal physiopathology in MSX2 mutant mice. Am J Pathol 2010;177(5):2516–2526. doi:10.2353/ajpath.2010.091224 [PubMed: 20934968]
19. Ruspita I, Das P, Xia Y, et al. An Msx2-Sp6-Follistatin Pathway Operates During Late Stages of Tooth Development to Control Amelogenesis. Front Physiol 2020;11:582610. Published 2020 Oct 26. doi:10.3389/fphys.2020.582610 [PubMed: 33192593]
20. Satokata I, Ma L, Ohshima H, et al. Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. Nat Genet 2000;24(4):391–395. doi:10.1038/74231 [PubMed: 10742104]
21. Zhou YL, Lei Y, Snead ML. Functional antagonism between Msx2 and CCAAT/enhancer-binding protein alpha in regulating the mouse amelogenin gene expression is mediated by protein-protein interaction. J Biol Chem 2000;275(37):29066–29075. doi:10.1074/jbc.M002031200 [PubMed: 10859305]
22. Berdal A, Hotton D, Pike JW, Mathieu H, Dupret JM. Cell- and stage-specific expression of vitamin D receptor and calbindin genes in rat incisor: regulation by 1,25-dihydroxyvitamin D3. Dev Biol 1993;155(1):172–179. doi:10.1006/dbio.1993.1016 [PubMed: 8380146]

23. Aïoub M, Lézot F, Molla M, et al. Msx2 −/− transgenic mice develop compound amelogenesis imperfecta, dentinogenesis imperfecta and periodental osteopetrosis. Bone 2007;41(5):851–859. doi:10.1016/j.bone.2007.07.023 [PubMed: 17878071]
24. Bolaños A, Hotton D, Ferbus D, Loiodice S, Berdal A, Babajko S. Regulation of calbindin-D(28k) expression by Msx2 in the dental epithelium. J Histochem Cytochem 2012;60(8):603–610. doi:10.1369/0022155412450641 [PubMed: 22614360]
25. Babajko S, de La Dure-Molla M, Jedeon K, Berdal A. MSX2 in ameloblast cell fate and activity. Front Physiol 2015;5:510. Published 2015 Jan 5. doi:10.3389/fphys.2014.00510 [PubMed: 25601840]
26. Nakatomi M, Ida-Yonemochi H, Nakatomi C, et al. Msx2 Prevents Stratified Squamous Epithelium Formation in the Enamel Organ. J Dent Res 2018;97(12):1355–1364. doi:10.1177/0022034518777746 [PubMed: 29863959]
27. Ruspita I, Miyoshi K, Muto T, Abe K, Horiguchi T, Noma T. Sp6 downregulation of follistatin gene expression in ameloblasts [published correction appears in J Med Invest. 2008 Aug;55(3–4):303]. J Med Invest 2008;55(1–2):87–98. [PubMed: 18319550]
28. Catón J, Luder HU, Zoupa M, et al. Enamel-free teeth: Tbx1 deletion affects amelogenesis in rodent incisors. Dev Biol 2009;328(2):493–505. doi:10.1016/j.ydbio.2009.02.014 [PubMed: 19233155]
29. Gibson CW, Yuan ZA, Hall B, et al. Amelogenin-deficient mice display an amelogenesis imperfecta phenotype. J Biol Chem 2001;276(34):31871–31875. doi:10.1074/jbc.M104624200 [PubMed: 11406633]
30. Zhou YL, Snead ML. Identification of CCAAT/enhancer-binding protein alpha as a transactivator of the mouse amelogenin gene. J Biol Chem 2000;275(16):12273–12280. doi:10.1074/jbc.275.16.12273 [PubMed: 10766866]
31. Gritli-Linde A, Bei M, Maas R, Zhang XM, Linde A, McMahon AP. Shh signaling within the dental epithelium is necessary for cell proliferation, growth and polarization. Development 2002;129(23):5323–5337. doi:10.1242/dev.00100 [PubMed: 12403705]
32. MacKenzie A, Ferguson MW, Sharpe PT. Expression patterns of the homeobox gene, Hox-8, in the mouse embryo suggest a role in specifying tooth initiation and shape. Development 1992;115(2):403–420. doi:10.1242/dev.115.2.403 [PubMed: 1358591]
33. Catron KM, Zhang H, Marshall SC, Inostroza JA, Wilson JM, Abate C. Transcriptional repression by Msx-1 does not require homeodomain DNA-binding sites. Mol Cell Biol 1995;15(2):861–871. doi:10.1128/MCB.15.2.861 [PubMed: 7823952]
34. Zhang H, Hu G, Wang H, et al. Heterodimerization of Msx and Dlx homeoproteins results in functional antagonism. Mol Cell Biol 1997;17(5):2920–2932. doi:10.1128/MCB.17.5.2920 [PubMed: 9111364]
35. Duval N, Daubas P, Bourcier de Carbon C, et al. Msx1 and Msx2 act as essential activators of Atoh1 expression in the murine spinal cord. Development 2014;141(8):1726–1736. doi:10.1242/dev.099002 [PubMed: 24715462]
36. Tian H, Lv P, Ma K, Zhou C, Gao X. Beta-catenin/LEF1 activated enamelin expression in ameloblast-like cells. Biochem Biophys Res Commun 2010;398(3):519–524. doi:10.1016/j.bbrc.2010.06.111 [PubMed: 20599763]
37. Chang H, Wang Y, Liu H, et al. Mutant Runx2 regulates amelogenesis and osteogenesis through a miR-185–5p-Dlx2 axis. Cell Death Dis 2017;8(12):3221. Published 2017 Dec 14. doi:10.1038/s41419-017-0078-4 [PubMed: 29242628]
38. Chu Q, Gao Y, Gao X, et al. Ablation of Runx2 in Ameloblasts Suppresses Enamel Maturation in Tooth Development. Sci Rep 2018;8(1):9594. Published 2018 Jun 25. doi:10.1038/s41598-018-27873-5 [PubMed: 29941908]
39. Athanassiou-Papaefthymiou M, Kim D, Harbron L, et al. Molecular and circadian controls of ameloblasts. Eur J Oral Sci 2011;119 Suppl 1(Suppl 1):35–40. doi:10.1111/j.1600-0722.2011.00918.x 42. [PubMed: 22243224]
40. Hu Y, Papagerakis P, Ye L, Feng JQ, Simmer JP, Hu JC. Distal cis-regulatory elements are required for tissue-specific expression of enamelin (Enam). Eur J Oral Sci 2008;116(2):113–123. doi:10.1111/j.1600-0722.2007.00519.x [PubMed: 18353004]

41. Zhang Z, Tian H, Lv P, et al. Transcriptional factor DLX3 promotes the gene expression of enamel matrix proteins during amelogenesis. PLoS One 2015;10(3):e0121288. Published 2015 Mar 27. doi:10.1371/journal.pone.0121288 [PubMed: 25815730]

42. Lézot F, Descroix V, Mesbah M, et al. Cross-talk between Msx/Dlx homeobox genes and vitamin D during tooth mineralization. Connect Tissue Res 2002;43(2–3):509–514. doi:10.1080/03008200290000583 [PubMed: 12489206]

43. Hu JC, Hu Y, Lu Y, et al. Enamelin is critical for ameloblast integrity and enamel ultrastructure formation. PLoS One 2014;9(3):e89303. Published 2014 Mar 6. doi:10.1371/journal.pone.0089303 [PubMed: 24603688]

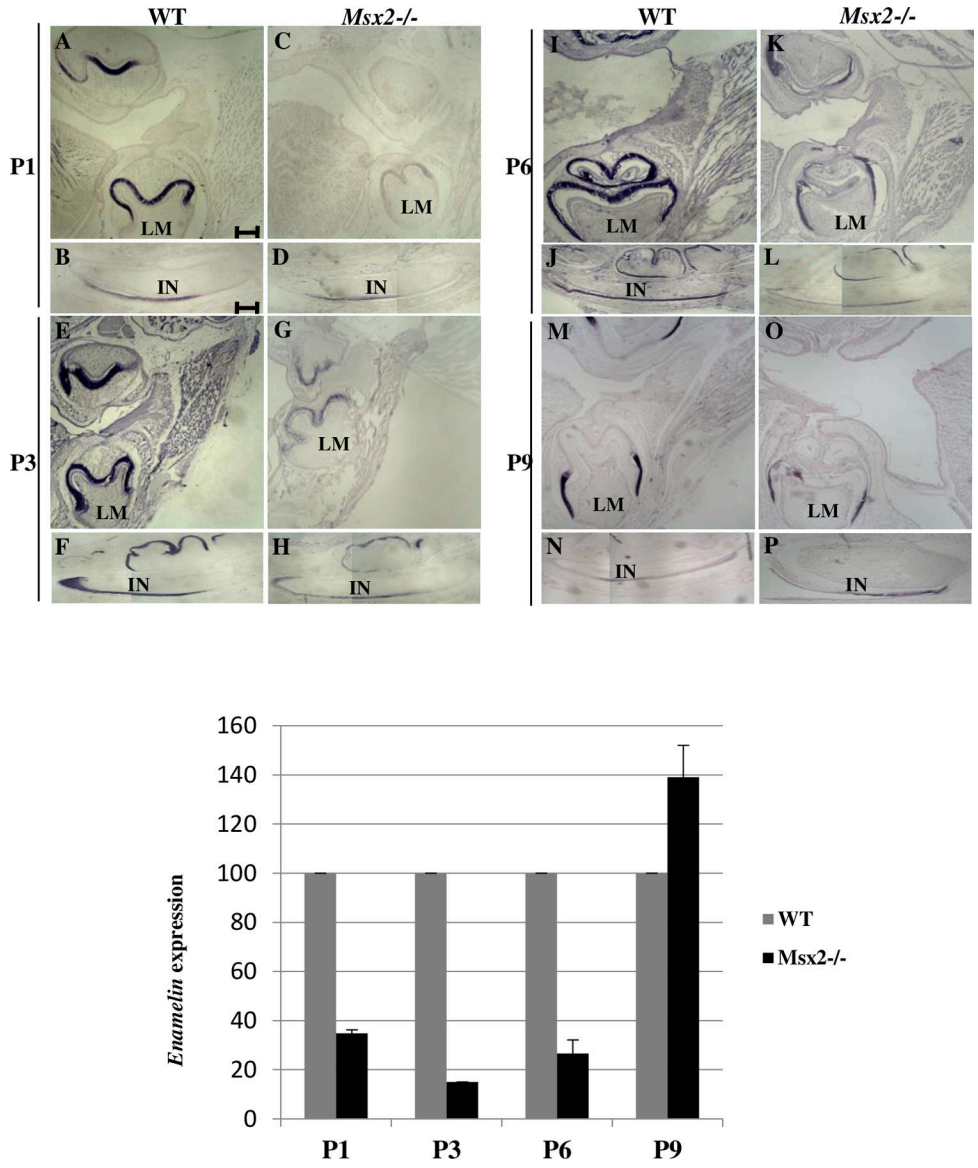

Fig. 1: Msx2 is essential for *enamelin* gene expression during amelogenesis:

In situ hybridization analyses of transcripts: **At postnatal day P1**: in wild type (A, B) and *Msx2* deficient (C, D) first lower molar teeth (A, C) and Incisors (B, D) **At postnatal day P3**: in wild type (E, F) and *Msx2* deficient (G, H) first lower molar teeth (E, F) and Incisors (G, H) **At postnatal day P6**: in wild type (I, J) and *Msx2* deficient (K, L) first lower molar teeth (I, K) and Incisors (J, L**At postnatal day P9**: in wild type (M, N) and *Msx2* deficient (O, P) first lower molar teeth (M, O) and Incisors (N, P) Expression of *Enamelin* is reduced in *Msx2* deficient ameloblasts compared to wild type at P1-P6 but not in P9. Abbreviations: LM: lower molars; IN: incisors. Scale: X100 (N=4).

Lower Panel: Total RNA from P1, P3, P6 and P9 tooth germs was extracted, reverse transcribed and qPCR was performed. *Enamelin* is downregulated in the *Msx2* deficient P1, P3 and P6 tooth germs but not at P9, further confirming our in-situ results. The higher expression of *enamelin* in the *Msx2* deficient teeth compared to wild type ones at P9 qPCR

is the result of the cumulative expression of *enamelin* from the buccal and lingual sides of the crown of the molars and from the proximal region of the crown of the incisors.

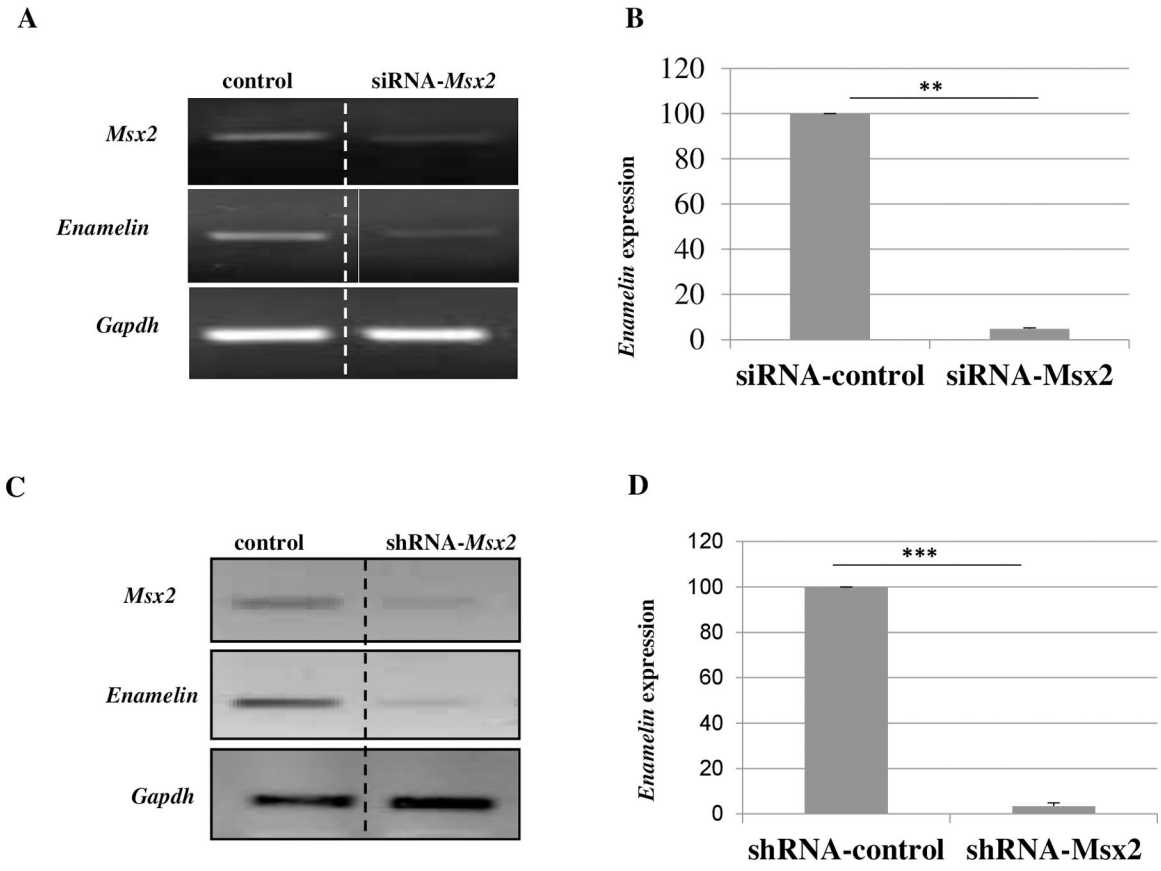

Fig. 2: Loss of function of *Msx2* in LS8 ameloblast-derived cells:

Expression of Enamelin after Msx2 knockdown using 2 different methods **(A)** siRNA mediated and **(C)** shRNA lentiviral-mediated to confirm downregulation of Msx2 transiently as well as long-term, respectively. **(B)** and **(D)** is the q-PCR to confirm the results as seen in (A) and (C). *Gapdh* is the normalizing gene. Experiments were done in triplicates. ** p=<0.005; ***p=<0.0001. Dotted line demarcates representative sample.

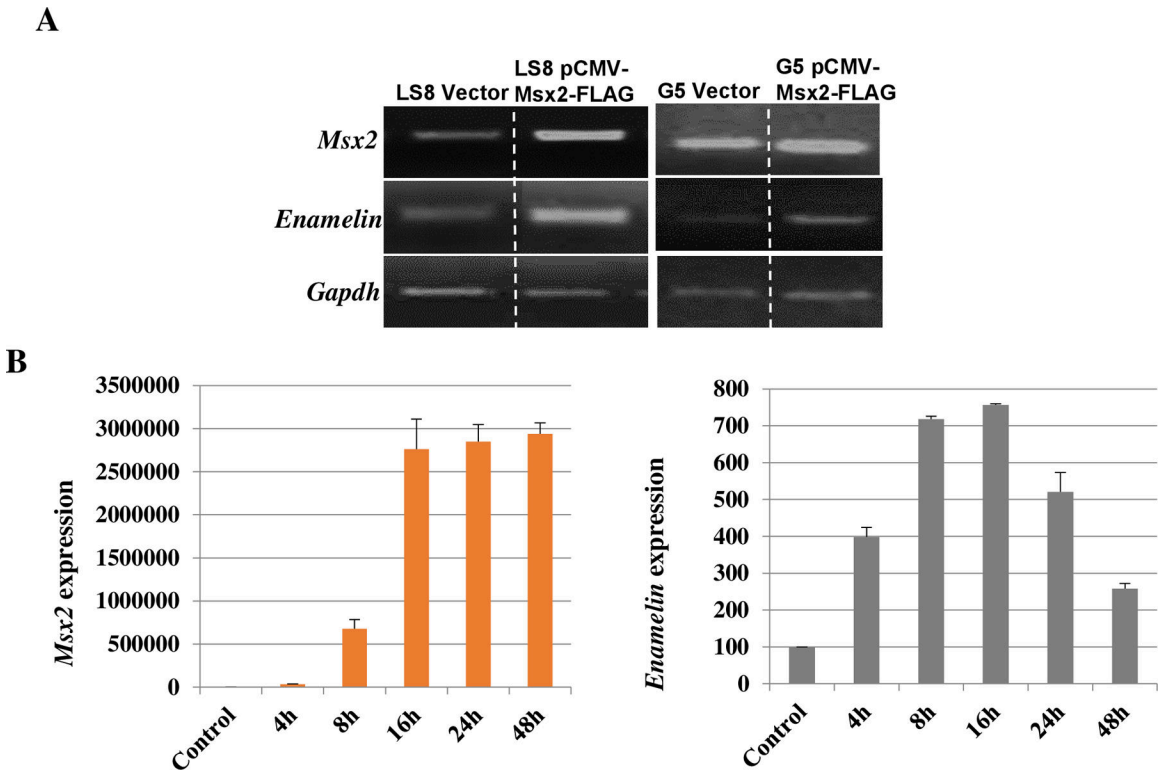

Fig. 3: The expression of *enamelin* is upregulated and modulated early in response to *Msx2:*

(**A**) Both LS8 and G5 cells were overexpressed with *Msx2* over-expression plasmid. Representative RT-PCR showing *enamelin* is upregulated in both cell lines after *Msx2* overexpression. (**B**) Time dependent Assay: LS8 cells were transfected with *Msx2* over-expression plasmid for several time points, 4, 8, 16, 24 and 48 hours. Total RNA was isolated from the cells and subjected to qPCR analysis. *Msx2* and *Enamelin* could be detected as early as 4h after transfection by real time qPCR. By 16h, there was a significant increase in the expression of *Msx2* with a corresponding increase of *Enamelin* expression. *Gapdh* is the normalizing gene. Bottom panel shows the expression of Msx2 and with corresponding upregulation of Enamelin in a logarithmic scale**,** after overexpression with Msx2 overexpression vector; **Control:** cells transfected with control vector only. The experiment was conducted 3 times in replicates of 3. Dotted line demarcates representative sample.

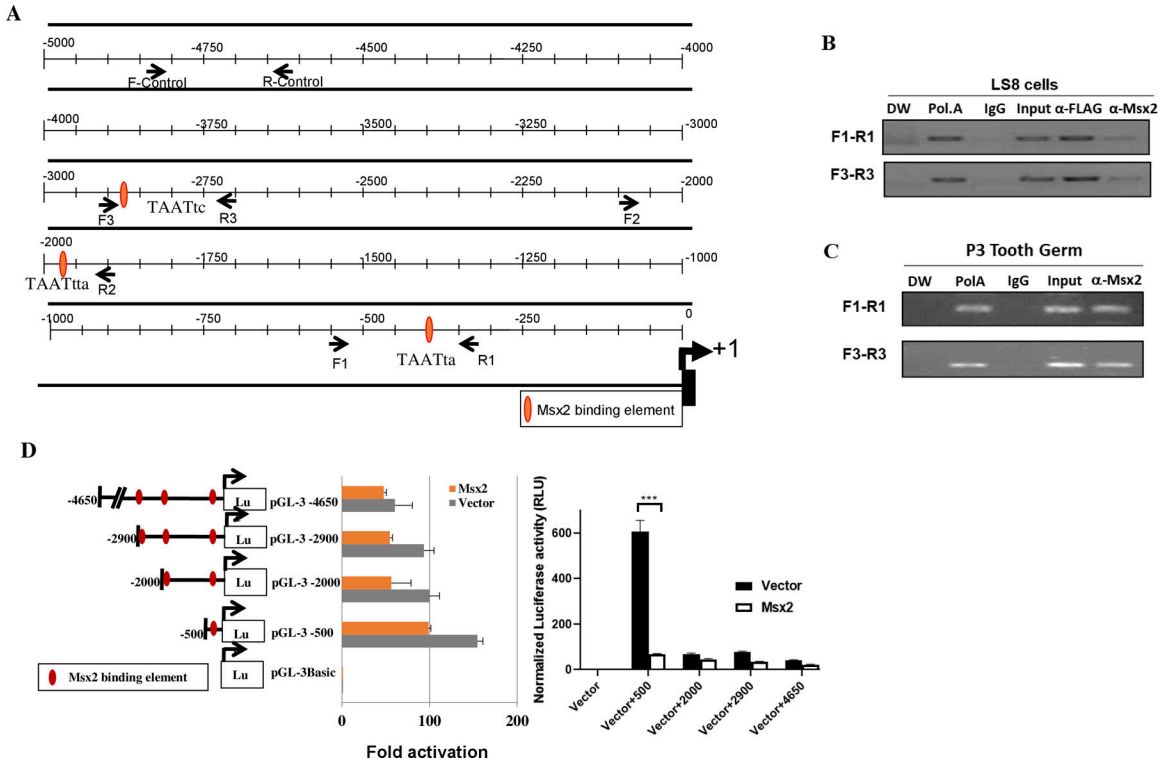

Fig. 4: Msx2 directly binds to *Msx2* recognition sites on the *Enam* promoter:

(A) *In silico* model showing three potential homeodomain binding sites (−500bp, −2000bp, −2900bp) on the *Enamelin* promoter using MatInspector. The different binding sites are represented by red ellipses. Primers were designed from different promoter regions (black arrows). (**B&C**) After chromatin immunoprecipitation, samples from LS8 cells transfected with pCMV-Msx2-FLAG and P3 wild type mouse M1 molar tooth germs were PCR amplified and the binding region was directly amplified prior to immunoprecipitation (1% Input) and specifically amplified in the immunoprecipitated sample (anti-FLAG) and (anti-Msx2). No amplification was detected in the normal mouse serum IgG-immunoprecipitated sample (IgG; negative control; DW: distilled water, negative control).). Pol A is the positive control. Band in the input lane shows endogenous binding while band in the sample lane shows binding after specific immunoprecipitation with FLAG tagged antibody and Msx2 antibody after overexpression. The results confirm the binding of *Msx2* to the predicted regions of the Enam promoter, both *in vitro* and *in vivo.* (**D**) LS8 cells were co-transfected with *enamelin*-luciferase reporter plasmids and *Msx2* expression plasmid. Cells were harvested 24h after transfection for reporter gene assays. Transcription efficiency was determined using *Renilla* luciferase plasmid. For this and subsequent experiments, the levels of luciferase activity were normalized to *Renilla* luciferase activity and expressed as fold luciferase activity relative to the level of luciferase activity from cells transfected with the reporter construct and empty expression plasmid. All the transfection experiments were performed three times, and results are shown as means +/− standard deviations. There was almost half-fold repression of *enamelin* activity in all the 3 regions that contained the putative binding sites. Right panel shows the quantification of the expression level

of luciferase for the vector relative to Msx2. The 500bp region shows a significant downregulation of Msx2. ***p<0.005; RLU: relative luminescence unit