

REPORT DOCUMENTATION PAGE			Form Approved OMB NO. 0704-0188		
The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA, 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 13-11-2019		2. REPORT TYPE Final Report		3. DATES COVERED (From - To) 1-Jul-2015 - 30-Sep-2019	
4. TITLE AND SUBTITLE Final Report: Organic-mediated Mineral Transport and Force Transduction in an Ultrahard Biological Composite: Biochemistry 8.1			5a. CONTRACT NUMBER W911NF-15-1-0306		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER 611102		
6. AUTHORS			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAMES AND ADDRESSES University of California - Riverside 200 University Office Building Riverside, CA 92521 -0001			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS (ES) U.S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211			10. SPONSOR/MONITOR'S ACRONYM(S) ARO		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S) 67448-LS-H.1		
12. DISTRIBUTION AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON David Kisailus
a. REPORT UU	b. ABSTRACT UU	c. THIS PAGE UU			19b. TELEPHONE NUMBER 951-827-4310

RPPR Final Report

as of 09-May-2020

Agency Code:

Proposal Number: 67448LSH

Agreement Number: W911NF-15-1-0306

INVESTIGATOR(S):

Name: David Kisailus

Email: kisailus@ucr.edu

Phone Number: 9518274310

Principal: Y

Organization: **University of California - Riverside**

Address: 200 University Office Building, Riverside, CA 925210001

Country: USA

DUNS Number: 627797426

EIN: 956006142

Report Date: 31-Dec-2019

Date Received: 13-Nov-2019

Final Report for Period Beginning 01-Jul-2015 and Ending 30-Sep-2019

Title: Organic-mediated Mineral Transport and Force Transduction in an Ultrahard Biological Composite: Biochemistry 8.1

Begin Performance Period: 01-Jul-2015

End Performance Period: 30-Sep-2019

Report Term: 0-Other

Submitted By: David Kisailus

Email: kisailus@ucr.edu

Phone: (951) 827-4310

Distribution Statement: 1-Approved for public release; distribution is unlimited.

STEM Degrees:

STEM Participants:

Major Goals: The overall objectives of the project are to investigate the nano- and microstructural features of the immature (i.e., non-mineralized and partially mineralized) chiton radular teeth as well as the supporting flexible stylus. These investigations will be combined with chemical (and phase) composition and mechanical properties in order to investigate the effects of the underlying organic framework on (i) providing pathways for mineral transport, (ii) templating mineral nucleation and growth and (iii) serving as a scaffold that interfaces a flexible organic structure with (and transduces force to) a stiff dense component. The information gleaned from these investigations will be used to provide both design and synthesis platforms for lightweight multifunctional composites. Based on our preliminary data, we believe that through the careful control of organic synthesis and hierarchical assembly, the local and global architectures of inorganic / organic composite can be modulated to increase the damage tolerance of these ultrahard materials, while providing utility.

Specifically, we will examine the ultrastructure of the stylus and partially mineralized teeth and the chemistry involved in the biomineral formation as outlined according to the following investigations:

1. An understanding of the structure and chemistry of the organic stylus and radular belt (in functional teeth) with a complementary mechanical investigation (combined modeling and experimental) of these complex structures that interfaces with mineralized teeth and enables force transduction.
2. Investigation of the mechanism of mineral transport within the stylus and its effect on the formation of the teeth.
3. A thorough investigation of the underlying organic framework (structural and chemical) that regulates nucleation and growth of ferrihydrite and its transformation to magnetite in the teeth.
4. Fabrication of biomimetic multifunctional structures through organic fiber assembly and in-vitro mineral transport studies to understand organic-mineral interactions and force transduction capabilities.

At the completion of this project, we have made significant progress towards our objectives, although we identified many more scientific questions as we progressed through this research.

Accomplishments: Based on the three years of this project as well as the additional no cost extension year, we have been largely successful in staying on schedule and achieving the milestones outlined in the proposal. In the past year, we have found more evidence about the mechanism of phase transformation from ferrihydrite to magnetite, likely from ferrous iron attaching to randomly ordered ferrihydrite and initiating both a change in solubility of the ferrihydrite as well as reduction of ferric iron species. In addition, we identified changes in phosphorus within the stylus, specifically at the junction zone. It appears that the junction zone phosphorous concentration rises sharply prior to tooth mineralization, perhaps acting as a barrier, to keep iron inside the tooth as the shell forms in stage 2 and 3 of mineralization. A second spike in phosphorous in the stylus occurs prior to core mineralization.

RPPR Final Report as of 09-May-2020

Beyond this, we constructed the first comprehensive transcriptome data from the radular tissue of the gumboot chiton, *Cryptochiton stelleri* (*C. stelleri*). Its formation mechanism has subsequently been of interest to molecular biologists as well as materials scientists. The constructed transcriptome data provides new insights into the genes highly expressed in radular tissue during the teeth formation in *C. stelleri*. Furthermore, by using the transcriptome data, a proteome profile of mineralized cusp-specific proteins was determined. These specific proteins include a novel protein that we term radular teeth matrix protein1 (RTMP1), globins, peroxidases, antioxidant enzymes and a ferroxidase protein. Those transcriptome and proteome data represent invaluable resources for the investigation of magnetite biomineralization (including iron oxide nucleation, growth and phase transformation) in chitons. Of great interest are other proteins such as Myoglobin I and Peroxiredoxin 6, both of which have potential to provide a reducing environment that could aid in modifying the environment for phase transformation to magnetite. The novel protein, RTMP 1, had a significant fraction of serine and glycine, which we showed was likely phosphorylated and could aid in nucleation of iron oxide species. Finally, 3D printed parts were made, mimicking the stylus, and tested under bending and twisting modes. Indeed, the geometry of the bulk structure is critical in controlling the stress needed to deform the stylus. This suggested that the multiscale hierarchy of the stylus is important in ensuring force can be transduced to rocky substrates.

Training Opportunities: Supported Personnel and Collaborations:
Training, Education, and Outreach Achievements
Training, Education, and Outreach Achievements
UCR:

1. Mr. Steven Herrera (BS, UCR and underrepresented minority), was a Ph.D. student working on the project. He was mentored by Kisailus.

2. Dr. Anna Pohl was a postdoc partially supported on this project. She was mentored by Kisailus.

We have had more than 7 groups of high school and community college students tour our lab in the past year.

Dr. Michiko Nemoto is a former Kisailus post-doc and now a collaborator in this project. She is now a professor at Okayama University. She was mentored as a professor by Kisailus.

Dr. Omar Avila-Poveda is a collaborator at the Universidad of Sinaloa in Mexico.

RPPR Final Report

as of 09-May-2020

Results Dissemination: Publications:

"Integrated transcriptomic and proteomic analyses of a molecular mechanism of radular teeth biomineralization in *Cryptochiton stelleri*," Michiko Nemoto, Dongni Ren, Steven Herrera, Songqin Pan, Takashi Tamura, Kenji Inagaki, David Kisailus, Scientific Reports, 9 (2019), 856. DOI: 10.1038/s41598-018-37839-2

Two additional manuscripts are in preparation.

Presentations:

"Biological Crystallization of Ultrahard Teeth and Translation to Multi-Functional Materials," Invited talk. 19th International Conference on Crystal Growth and Epitaxy (ICCGE-19). Keystone, Colorado, July 28–August 2, 2019.

"Biological Crystallization of Ultrahard Teeth and Translation to Multi-Functional Materials," Invited talk. Collaborative Conference on Materials Research. Seoul, South Korea. June 25-29, 2019.

"Biologically Inspired Multi-Functional Materials," Invited seminar. Lawrence Berkeley National Labs. Berkeley, CA. April 17, 2019.

"Biological Crystallization of Ultrahard Teeth and Translation to Multi-Functional Materials," Invited talk. 43rd International Conference and Exposition on Advanced Ceramics Conference, Daytona Beach, FL, January, 2019.

"Biologically Inspired Multi-Functional Materials For Structural, Energy and Water-Related Applications", Invited seminar. Chemistry Department, Sun Yat Sen University. Guangzhou, China. December, 2018.

"Biologically Inspired Multi-Functional Materials," Invited seminar. Aerospace Engineering Department, University of Illinois at Urbana-Champaign. Urbana, IL. October 29, 2018.

"Materials Inspired from Fossils and Their Relatives," Invited talk. Materials Science and Engineering Congress (MSE). Darmstadt, Germany. September 26-28, 2018.

"Fiber Functionality in Nature and Beyond: Assembly, Templating and Toughening," Keynote lecture. Global Innovation Research Symposium. Tokyo, Japan. September 20, 2018.

"Biological Synthesis and Structural Developments in the Ultrahard Teeth of Chiton," Invited talk. 6th International Meeting on Magnetotactic Bacteria. Kanazawa, Japan. September 10-14, 2018.

"Multifunctional Materials for Structural, Energy and Water Related Applications," Invited talk. International Materials Research Congress. Cancun, Mexico. August 21, 2018.

Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: Nothing to Report

PARTICIPANTS:

Participant Type: PD/PI

Participant: David Kisailus

Person Months Worked: 3.00

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Funding Support:

RPPR Final Report
as of 09-May-2020

Participant Type: Co-Investigator

Participant: Michiko Nemoto

Person Months Worked: 1.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Co-Investigator

Participant: Omar Avila-Poveda

Person Months Worked: 1.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Graduate Student (research assistant)

Participant: Steven Herrera

Person Months Worked: 2.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)

Participant: Anna Pohl

Person Months Worked: 2.00

Funding Support:

Project Contribution:

International Collaboration:

International Travel:

National Academy Member: N

Other Collaborators:

ARO Final Report (2019)

Organic-mediated Mineral Transport and Force Transduction in an Ultrahard Biological Composite

PI: David Kisailus, UC Riverside

Program Manager: Dr. Stephanie McElhinny, Biochemistry, Life Sciences Division

Abstract

Based on the three years of this project as well as the additional no cost extension year, we have been largely successful in staying on schedule and achieving the milestones outlined in the proposal. In the past year, we have found more evidence about the mechanism of phase transformation from ferrihydrite to magnetite, likely from ferrous iron attaching to randomly ordered ferrihydrite and initiating both a change in solubility of the ferrihydrite as well as reduction of ferric iron species. In addition, we identified changes in phosphorus within the stylus, specifically at the junction zone. It appears that the junction zone phosphorous concentration rises sharply prior to tooth mineralization, perhaps acting as a barrier, to keep iron inside the tooth as the shell forms in stage 2 and 3 of mineralization. A second spike in phosphorous in the stylus occurs prior to core mineralization. Beyond this, we constructed the first comprehensive transcriptome data from the radular tissue of the gumboot chiton, *Cryptochiton stelleri* (*C. stelleri*). Its formation mechanism has subsequently been of interest to molecular biologists as well as materials scientists. The constructed transcriptome data provides new insights into the genes highly expressed in radular tissue during the teeth formation in *C. stelleri*. Furthermore, by using the transcriptome data, a proteome profile of mineralized cusp-specific proteins was determined. These specific proteins include a novel protein that we term radular teeth matrix protein1 (RTMP1), globins, peroxidases, antioxidant enzymes and a ferroxidase protein. Those transcriptome and proteome data represent invaluable resources for the investigation of magnetite biomineralization (including iron oxide nucleation, growth and phase transformation) in chitons. Of great interest are other proteins such as Myoglobin I and Peroxiredoxin 6, both of which have potential to provide a reducing environment that could aid in modifying the environment for phase transformation to magnetite. The novel protein, RTMP 1, had a significant fraction of serine and glycine, which we showed was likely phosphorylated and could aid in nucleation of iron oxide species. Finally, 3D printed parts were made, mimicking the stylus, and tested under bending and twisting modes. Indeed, the geometry of the bulk structure is critical in controlling the stress needed to deform the stylus. This suggested that the multiscale hierarchy of the stylus is important in ensuring force can be transduced to rocky substrates.

Overall objectives

The overall objectives of the project are to investigate the nano- and microstructural features of the immature (i.e., non-mineralized and partially mineralized) chiton radular teeth as well as the supporting flexible stylus. These investigations will be combined with chemical (and phase) composition and mechanical properties in order to investigate the effects of the underlying organic framework on (i) providing pathways for mineral transport, (ii) templating mineral nucleation and growth and (iii) serving as a scaffold that interfaces a flexible organic structure with (and transduces force to) a stiff dense component. The information gleaned from these investigations will be used to provide both design and synthesis platforms for lightweight multifunctional composites. Based on our preliminary data, we believe that through the careful control of organic synthesis and hierarchical assembly, the local and global architectures of inorganic / organic composite can be modulated to increase the damage tolerance of these ultrahard materials, while providing utility.

Specifically, we will examine the ultrastructure of the stylus and partially mineralized teeth and the chemistry involved in the biomineral formation as outlined according to the following investigations:

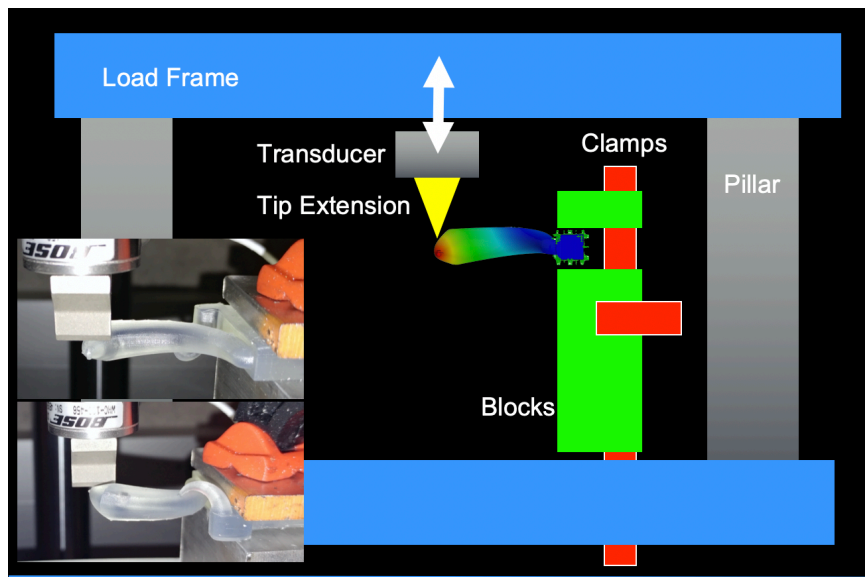
1. An understanding of the structure and chemistry of the organic stylus and radular belt (in functional teeth) with a complementary mechanical investigation (combined modeling and experimental) of these complex structures that interfaces with mineralized teeth and enables force transduction.
2. Investigation of the mechanism of mineral transport within the stylus and its effect on the formation of the teeth.
3. A thorough investigation of the underlying organic framework (structural and chemical) that regulates nucleation and growth of ferrihydrite and its transformation to magnetite in the teeth.
4. Fabrication of biomimetic multifunctional structures through organic fiber assembly and in-vitro mineral transport studies to understand organic-mineral interactions and force transduction capabilities.

At the completion of this project, we have made significant progress towards our objectives, although we identified many more scientific questions as we progressed through this research.

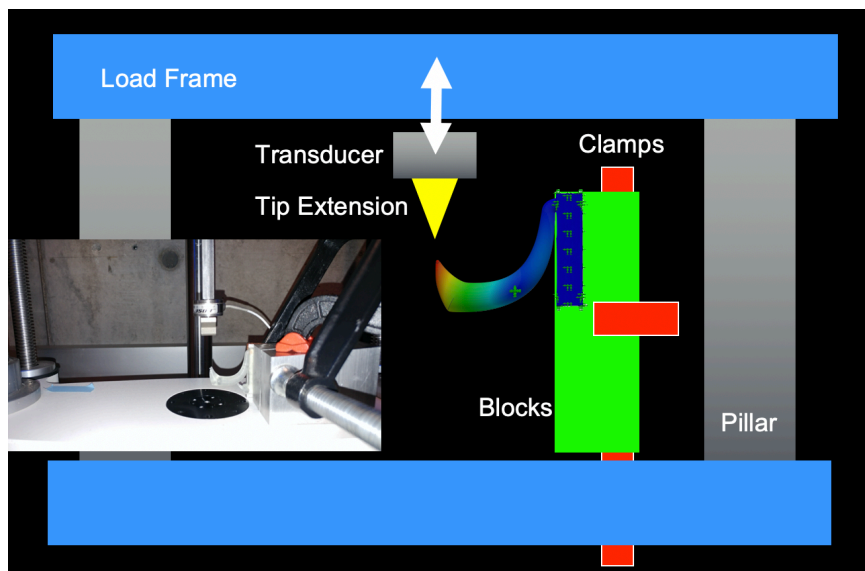
Accomplishments of Specific objectives:

1. An understanding of the structure and chemistry of the organic stylus and radular belt (in functional teeth) with a complementary mechanical investigation (combined modeling and experimental) of these complex structures that interfaces with mineralized teeth and enables force transduction.

During the past year, we used microscopic and spectroscopic techniques to investigate the tooth development from both the chiton stylus and within the teeth. Here, we 3D printed styli with varied geometrical features – specifically the shape of the cross section of the stylus.

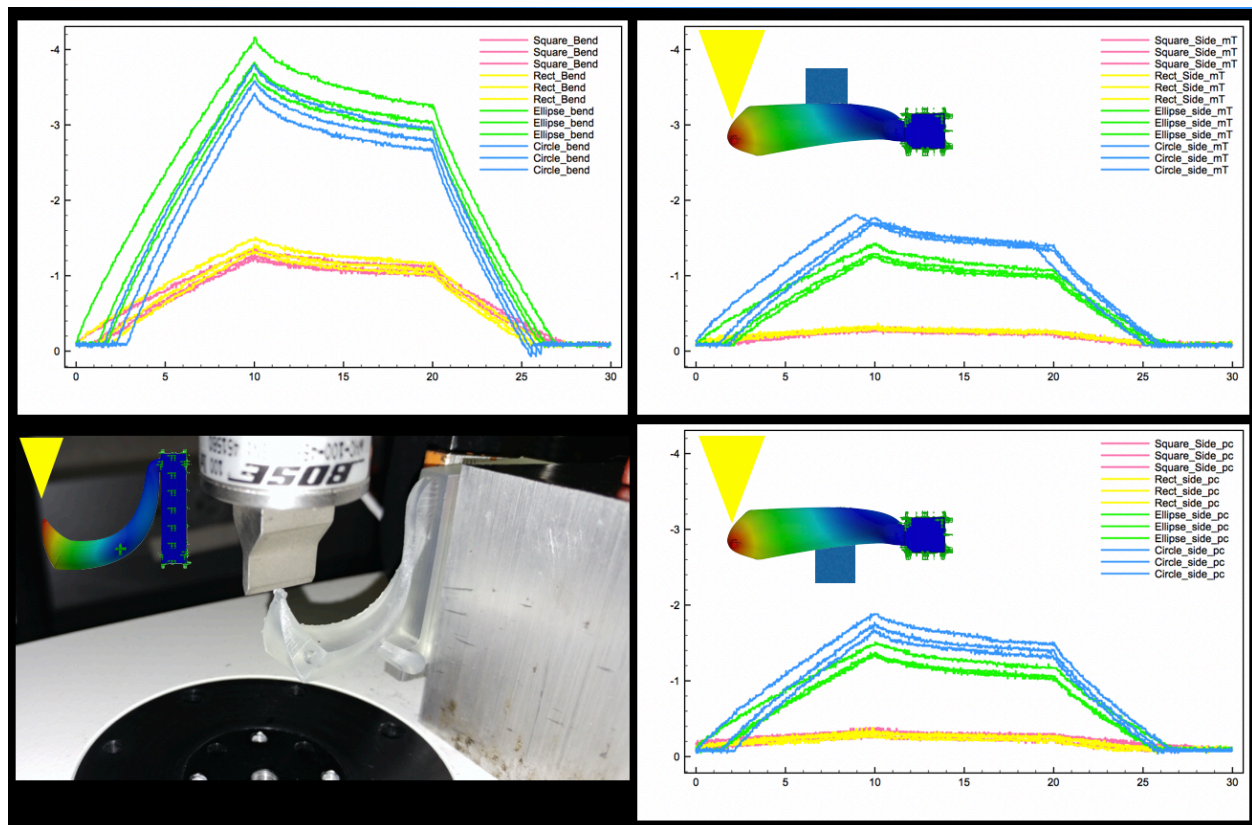


[Mechanical testing of stylus under twisting mode.]



[Mechanical testing of stylus under bending mode.]

In both sets of tests, the stylus printed models were clamped to the lower platform. The transducer was mounted to the stepper motor above and controlled displacement was performed with a force range of 15-50 N.



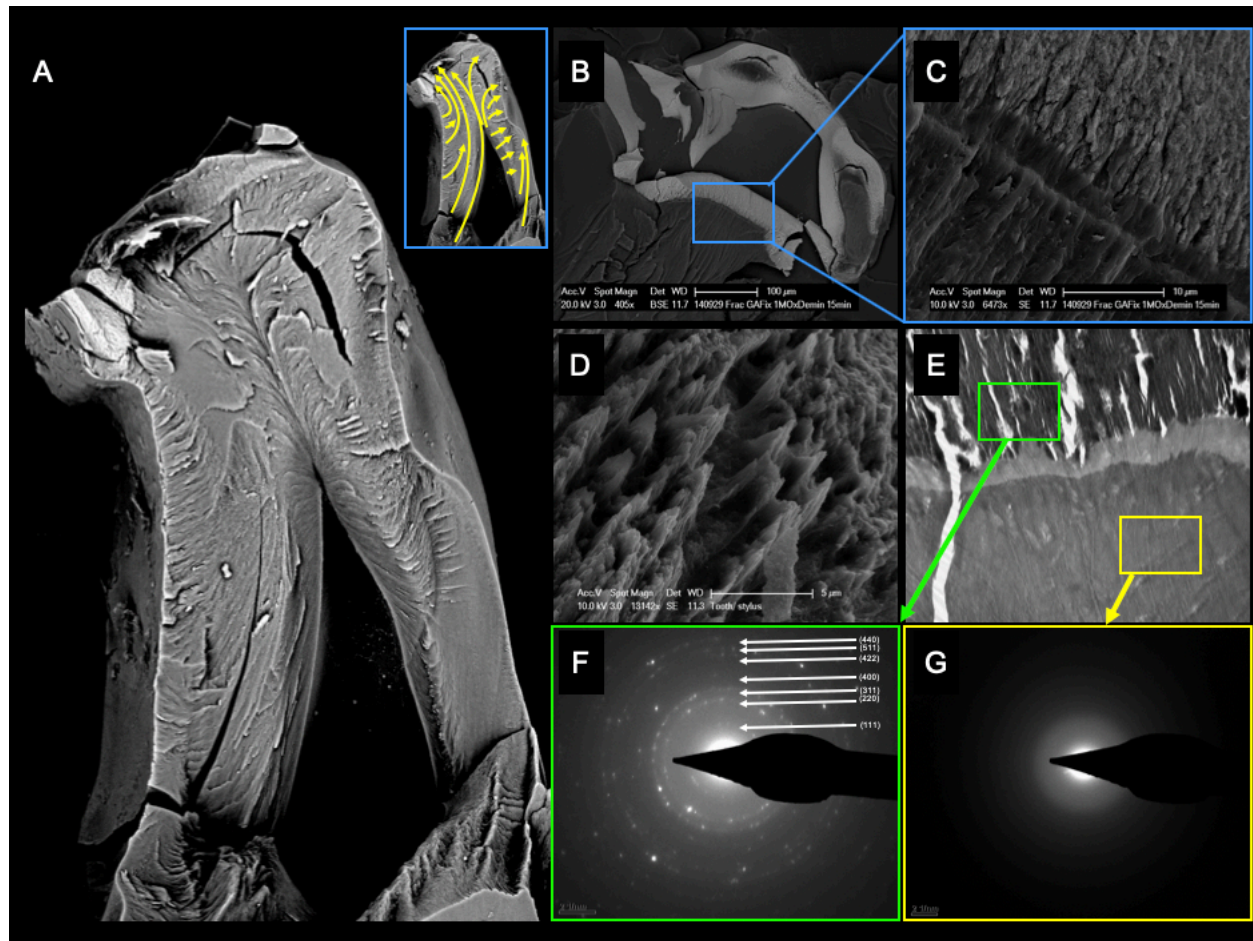
[Mechanical test results in bending mode for 3D printed styli.]

Interestingly, both elliptical and circular cross sections, which are more similar to the natural design in the chiton, yielded a larger stress required for bending, indicating their effectiveness in providing force transduction to the rocky substrates. Interestingly, both rectangular and square geometries did not show substantial forces upon bending. Additional analysis is needed here, but it is clear that nature has selected the optimal geometry for function.

In the future, more work has to be done on the analysis of the forces required to bend the stylus, specifically focusing on the role of the inner geometry of the stylus (a hollow tube), and also the material properties. This is due to the fact that the stylus is a graded composite, with elements such as sulfur placed in regions where stress seems to be higher. Similar to a vulcanized rubber, which utilizes sulfur to cross link rubber molecules, it is likely that the sulfur we observe in the stylus comes from either / both disulfide bonds or sulfate groups, which may inhibit crystallization of iron during transport within the stylus to the teeth.

Beyond the geometrical features of the stylus, we were interested in observing the microstructural features of the stylus, and their role in providing localized stiffness as well as strength. In addition, as our work suggested transport of mineral from the stylus to the teeth, it is likely that there are conduits within the stylus for mineral transport. Thus, after preparing a number of fractured

samples as well as embedded/sectioned samples, we have a clearer idea about the microstructural designs within the stylus.



[Fractures and microtomed sections of the stylus.]

Our analyses revealed highly ordered fiber sheets with a complex layup. In the above figure: A. Longitudinal fracture section along the mid plane of the stylus, a small remnant of the tooth remains on the leading edge (higher contrast) at the junction zone. The inset maps highlight the fiber direction in this cross section. B. Coronal fracture of the tooth across the junction zone reveals C. fibers transcending the stylus and passing through the junction zone into the shell of the tooth. D. A transverse fracture at the junction zone shows well-arranged fibers passing through the junction into the shell of the tooth. E. TEM section of the junction zone. F. SAED of the green box region reveals diffraction patterns for magnetite and G. chitin in the yellow boxed region.

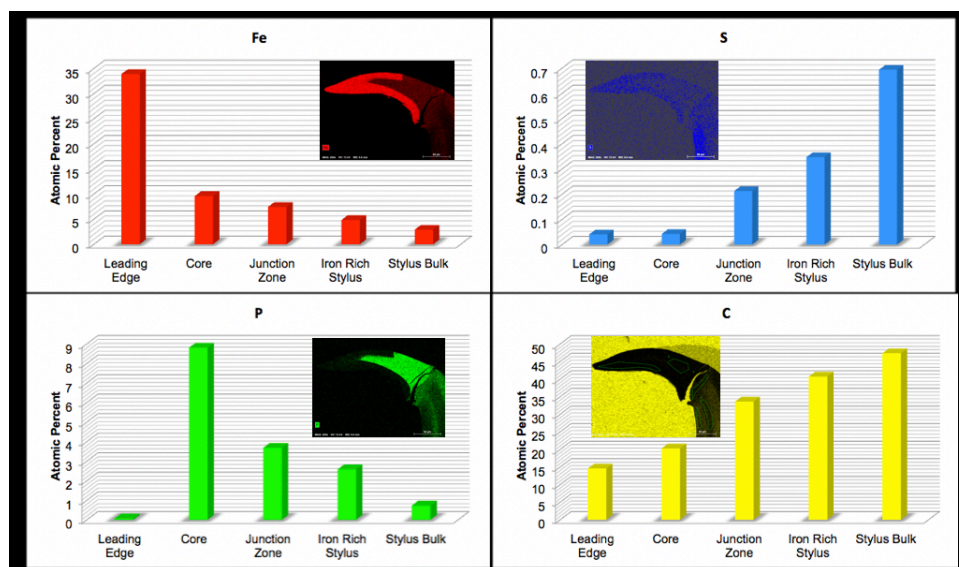
Although we have observed this fiber orientation, future work must involve printing or laying up fiber-reinforced styli to capture both geometry and microstructural effects.

Objectives 2 and 3.

2. Investigation of the mechanism of mineral transport within the stylus and its effect on the formation of the teeth.

3. A thorough investigation of the underlying organic framework (structural and chemical) that regulates nucleation and growth of ferrihydrite and its transformation to magnetite in the teeth.

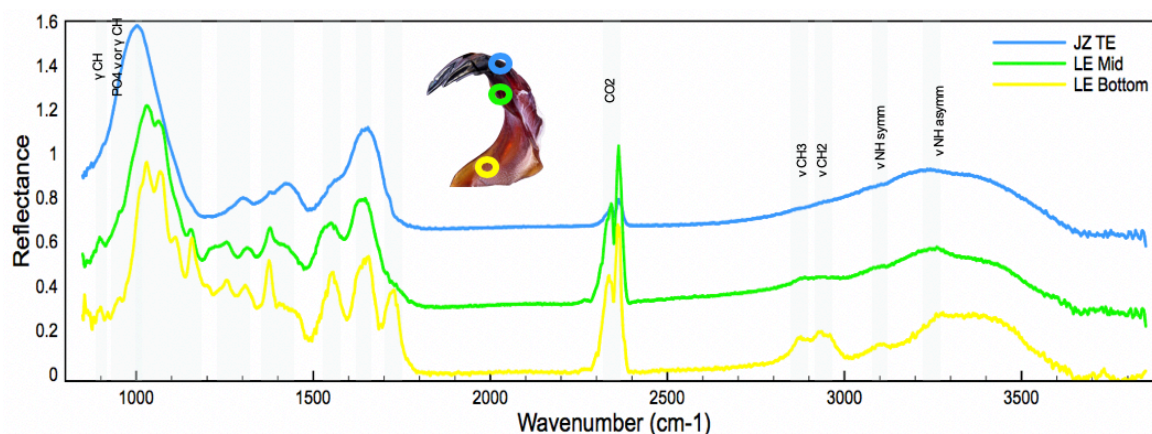
We have performed additional EDS mapping on different regions of the stylus and teeth. Specifically, we focused on Fe, S, P and C in the leading edge of the tooth, core of the tooth, junction zone between the stylus and tooth, the leading edge of the stylus leading into the bulk region of the tooth shell (“Iron rich stylus”) and the bulk of the stylus.



[EDS mapping of Fe, S, P and C in tooth and stylus of chiton.]

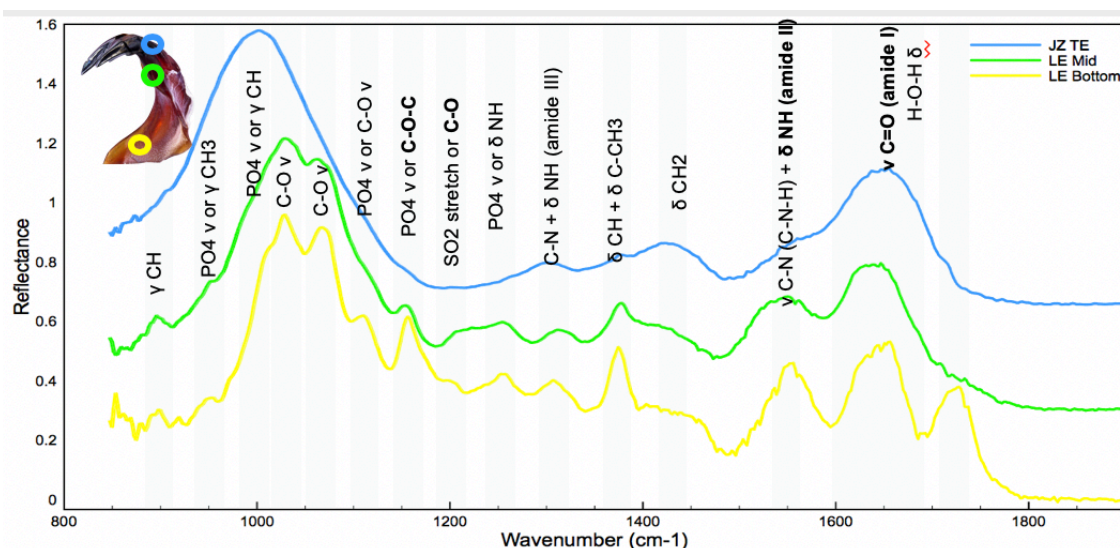
The fact that we see significant quantities of S and P in the stylus, specifically in the region where mineral (iron) may be transported into the bulk of the tooth, we hypothesize that these elements may be present in sulfated or phosphorylated forms that help to prevent nucleation of mineral during transport.

A significant effort was made towards understanding the chemistry of the stylus, which not only would control mineral transport to the tooth, but also the regional stiffness of the stylus that yields specific elastic moduli to control the mechanical behavior (and thus, also part of Objective 1). Here, we utilized a combination of nuclear magnetic resonance (for carbon and phosphorus), FTIR and synchrotron x-ray analysis of different regions of the tooth-stylus: (i) the junction zone on the trailing edge (JZ TE), where significant stress would develop during rasping, (ii) the leading edge middle of the stylus (LE Mid), where mineral is likely passing on its way to the bulk region of the tooth and (iii) the leading edge bottom of the stylus (LE bottom), where the stylus begins to connect with the radular belt. FTIR analysis of these 3 different regions highlighted some significant differences in their chemical compositions.



[Overall FTIR spectrum of 3 regions from the stylus and tooth.]

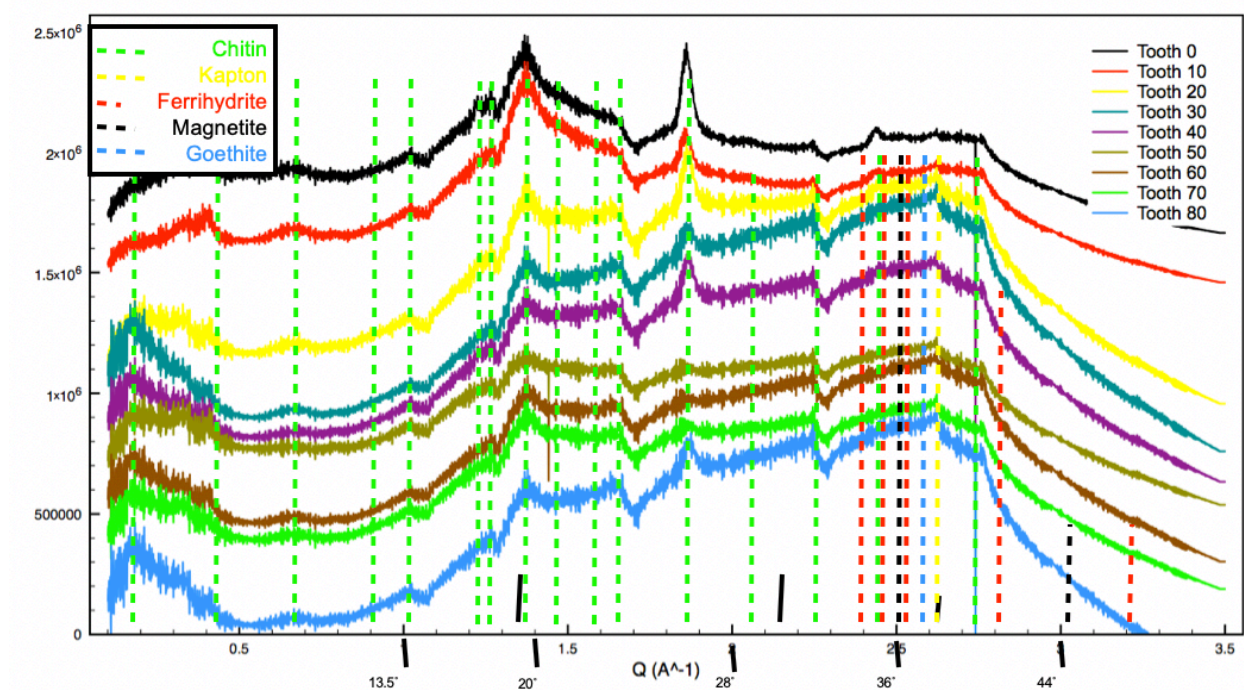
Analysis of this spectrum from 800 – 1800 cm^{-1} showed more detail.



[FTIR spectrum of stylus and tooth at junction zone, with peak identification.]

Our analysis highlights that there is phosphate groups in the stylus, and although not evaluated in significant detail yet, the presence of S=O groups is highly likely, suggesting that regions within the stylus are resistant to nucleation of iron, enabling precursors to be transported to the teeth effectively. Unfortunately, identification of S-H and S-S was difficult, as those peaks are seen from 500 – 700 cm^{-1} . In addition, they are weakly excited in FTIR mode. Future work will have to involve FTIR mapping as well as coordination with Raman.

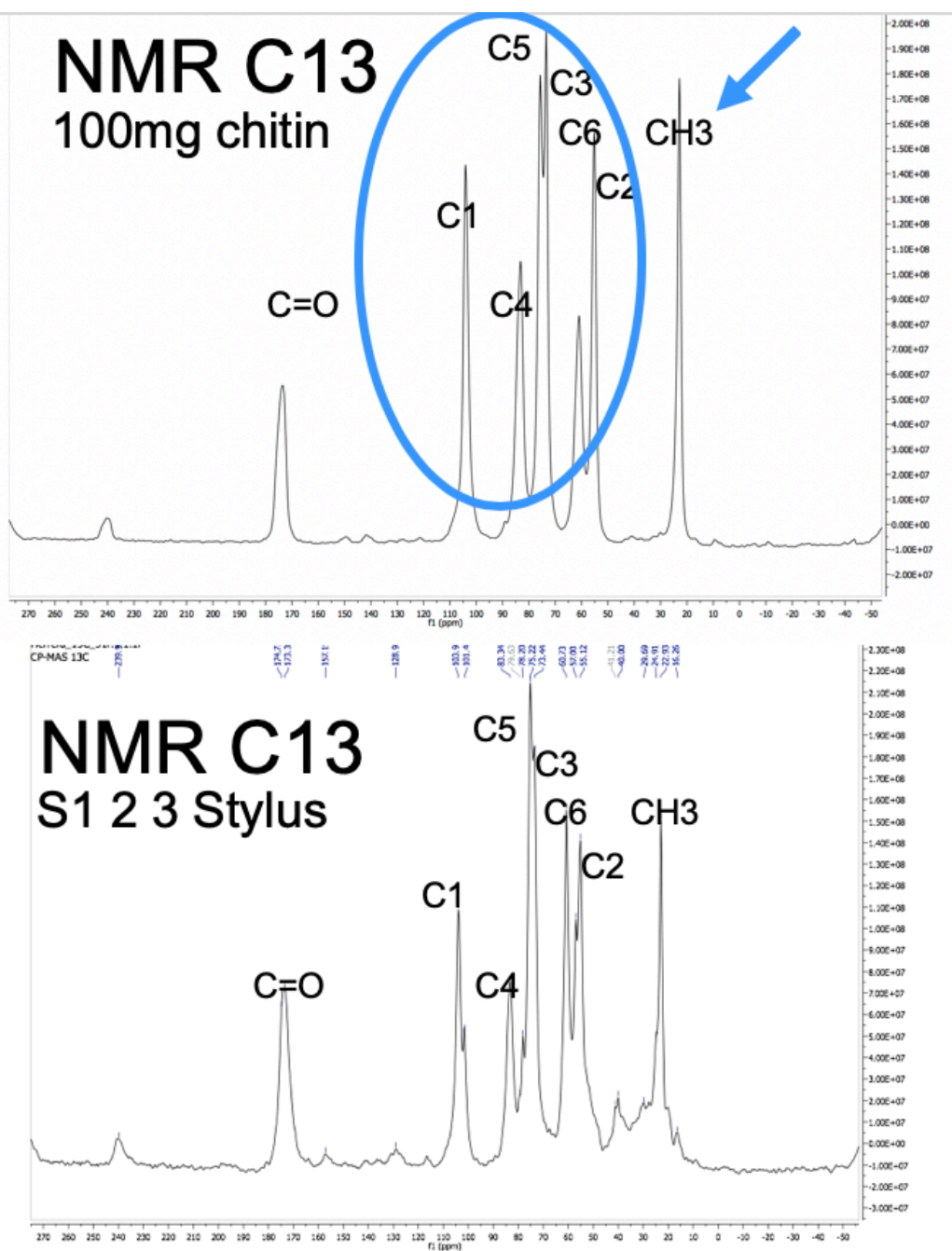
To check for mineral in the stylus, we conducted synchrotron wide angle x-ray scattering experiments (WAXS) on the stylus in the following rows of teeth: 0, 10, 20, 30, 40, 50, 60, 70 and 80.



[Synchrotron WAXS results from stylus in different rows.]

The *vertical* dotted lines represent reflections for alpha-chitin (green), kapton tape for holding the sample (yellow), ferrihydrite (red), magnetite (black) and goethite (light blue). It is clear from the diffraction patterns that no significant quantities of mineral are present, only reflections from alpha-chitin. Thus, our previous EDS results that show iron, as well as this WAXS data suggest that the iron is in an amorphous or complexed (potentially with proteins) state. Further investigation is needed here.

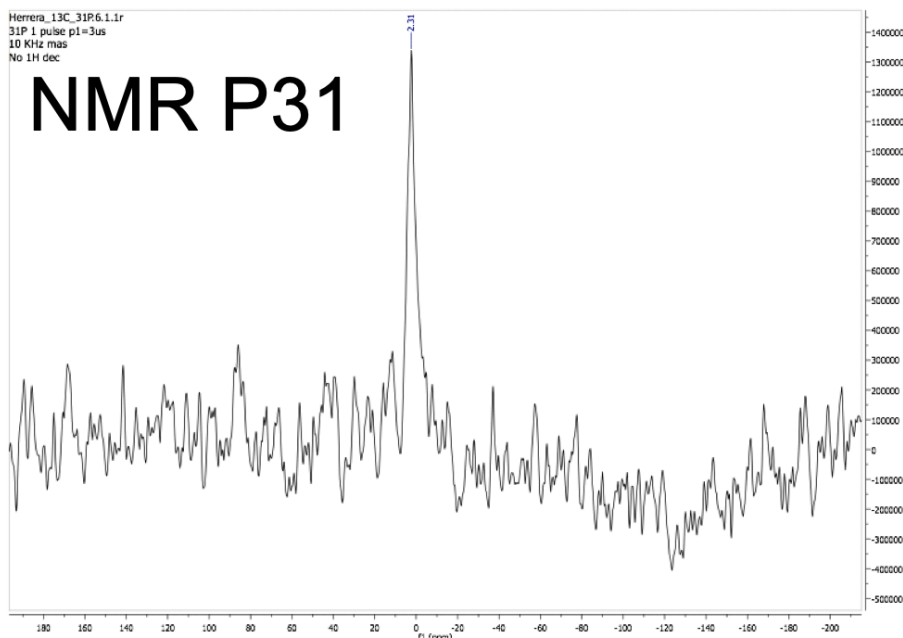
In addition, the EDS data highlighted discrete channels of iron in the stylus that were correlated with phosphorous. Also, Sulfur, although present in very low concentrations, has a strong negative correlation with iron, phosphorous, and calcium. Sulfur was present in very low concentrations. Since there is the presence of chitin throughout the structure nmr was conducted to try to elucidate the potential of a post-translationally modified chitin with sulfur or phosphorus. In this nmr work, we compared chitin from squid pen to the stylus from the chiton. We also wanted to identify if chitosan was present in the stylus.



[Carbon nmr of chitin control (top) and stylus (bottom).]

Here, it seems that in the stylus, the C_{13} NMR ratio of $CH_3 / (C1-6) = 1.02$. This suggests that that chitosan is not present in significant amounts. However, there are many additional peaks that have not been identified and require more analysis. This will be conducted in a future project.

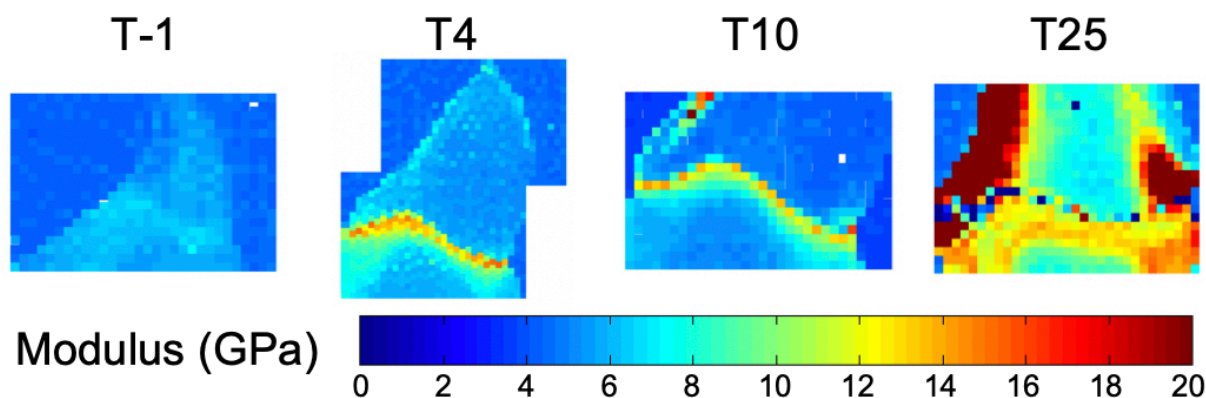
In addition, P_{31} nmr was conducted on the stylus.



[P₃₁ nmr on the stylus from chiton.]

Here, it seems that there is a P₃₁ NMR peak at 2.31 ppm. Possible configurations include: O=PX₃, O=P(OR)X₂, O=P(OR)₂X, O=P(R)(OR)₂, O=P(R)(OH)₂, (RO)₂POH, R₂P(=O)OR, R₄PX. Since FTIR suggests the presence of PO₄, we are looking to confirm that is actually P-O and not a form of P-C. Future work will address this.

Finally, we repeated some mechanical testing (nanoindentation) to measure the stiffness of the junction zone.



[Nanoindentation maps of the junction zone between tooth and stylus at different rows.]

These results show that there is a significant increase in stiffness between non-mineralized teeth (“T-1”) and teeth where mineralization has been initiated (“T4”). This suggests that mineral is being transported from the stylus through to the teeth.

Finally, we constructed the first comprehensive transcriptome data from the radular tissue of the gumboot chiton, *Cryptochiton stelleri* (*C. stelleri*). Its formation mechanism has subsequently been

of interest to molecular biologists as well as materials scientists. The constructed transcriptome data provides new insights into the genes highly expressed in radular tissue during the teeth formation in *C. stelleri*. Furthermore, by using the transcriptome data, a proteome profile of mineralized cusp-specific proteins was determined. These specific proteins include a novel protein that we term radular teeth matrix protein1 (RTMP1), globins, peroxidases, antioxidant enzymes and a ferroxidase protein. Those transcriptome and proteome data represent invaluable resources for the investigation of magnetite biomineralization (including iron oxide nucleation, growth and phase transformation) in chitons. Of great interest are other proteins such as Myoglobin I and Peroxiredoxin 6, both of which have potential to provide a reducing environment that could aid in modifying the environment for phase transformation to magnetite. The novel protein, RTMP 1, had a significant fraction of serine and glycine, which we showed was likely phosphorylated and could aid in nucleation of iron oxide species.

The following text is from the paper that we submitted and was accepted into Scientific Advances in early 2019.

Integrated transcriptomic and proteomic analyses of a molecular mechanism of radular teeth biomineralization in *Cryptochiton stelleri*

Michiko Nemoto^{1*}, Dongni Ren², Steven Herrera², Songqin Pan³, Takashi Tamura¹, Kenji Inagaki¹ and

David Kisailus^{2,4*}

¹ Graduate School of Environmental and life Science, Okayama University, Okayama 700-8530, Japan;

² Department of Chemical and Environmental Engineering, University of California, Riverside, CA, 92521, USA;

³ W. M. Keck Proteomics Laboratory, Institute for Integrative Genome Biology, University of California, Riverside, CA 92521, USA;

⁴ Materials Science and Engineering Program, University of California, Riverside, CA, 92521, USA;

* Correspondence: mnemoto@okayama-u.ac.jp; david@engr.ucr.edu

Abstract

Many species of chiton are known to deposit magnetite (Fe_3O_4) within the cusps of their heavily mineralized and ultrahard radular teeth. Recently, much attention has been paid to the ultrastructural design and superior mechanical properties of these radular teeth, providing a promising model for the development of novel abrasion resistant materials. Here, we constructed *de novo* assembled transcripts from the radular tissue of *C. stelleri* that were used for transcriptome and proteome analysis.

Transcriptomic analysis revealed that the top 20 most highly expressed transcripts in the non-mineralized teeth region include the transcripts encoding ferritin, while those in the mineralized teeth region contain a high proportion of mitochondrial respiratory chain proteins. Proteomic analysis identified 22 proteins that were specifically expressed in the mineralized cusp. These specific proteins include a novel protein that we term radular teeth matrix protein1 (RTMP1), globins, peroxidasins, antioxidant enzymes and a ferroxidase protein. This study reports the first *de novo* transcriptome assembly from *C. stelleri*, providing a broad overview of radular teeth mineralization. This new transcriptomic resource and the proteomic profiles of mineralized cusp are valuable for further investigation of the molecular mechanisms of radular teeth mineralization in chitons.

Introduction

Biologically formed magnetite (Fe_3O_4) has been found from several organisms including bacteria, chiton, homing pigeons, honeybees and salmon¹⁻⁵. In the last few decades, significant information has been accumulated regarding bacterial magnetite formation⁶⁻⁹. In contrast, little is known about molecular mechanisms of magnetite biomineralization in eukaryotes. The magnetite-rich teeth of chitons was first reported in 1962 and its formation mechanism has been of interest for several decades¹. Chitons have several dozen rows of teeth that are attached to a ribbon-like structure called a radular membrane. Each tooth is composed of a mineralized cusp and base (also referred to as the stylus) supporting the mineralized cusp (Fig. 1b). Magnetite is deposited only in the cusp region. Recently, ultrastructural and mechanical characterization of fully mineralized chiton teeth from *Cryptochiton stelleri* showed that it has the maximum hardness and stiffness of any known biomineral^{10,11}. Although some preliminary analysis provides critical structure-mechanical property relationships in these teeth^{12,13}, a further understanding of the formation machinery used to construct this ultrahard biomaterial will allow us to develop a novel abrasion resistant material.

We have studied magnetite biomineralization in the world's largest species of chiton, *C. stelleri*. As other chitons, *C. stelleri* use their teeth to scrape the algae growing on intertidal rocks. When these teeth become worn, they are replaced by a new row of teeth that are continuously formed from inside a radular sac¹⁴. Therefore, it is possible to observe all stages of tooth development within one radula. In the radular teeth of *C. stelleri*, no mineral deposits are found on the first 8 - 12 rows of teeth. These non-mineralized teeth are transparent and are mainly composed of α -chitin and proteins. The next 2 – 5 rows of teeth turn a reddish-brown color, representing a partial mineralization of the teeth with an amorphous or weakly nanocrystalline iron oxide (ferrihydrite). The subsequent rows of teeth have black-colored cusps, indicative of a transformation from ferrihydrite to magnetite towards fully mineralized teeth. Detailed observations of developing teeth revealed that the mineral aggregates of ferrihydrite first deposited on alpha-chitin fibers continuously grow in size as teeth maturation proceeds. Phase transformation to magnetite occurred within a few rows of teeth after the onset of ferrihydrite formation.

Although radular membrane, base and cusp are all composed of alpha-chitin, iron-based mineral was deposited only in the cusp region. From these results, we assume that there may be several proteins that dictate specific iron oxide nucleation in the cusp region as well as transformation to magnetite in chiton teeth^{15,16}. To reveal the proteins involved in the formation of radular teeth, we previously conducted proteome analysis of mineralized teeth of *C. stelleri* using a *de novo* peptide sequencing technology¹⁵. However, because of the lack of genomic sequence information, only partial sequences of mineralized teeth proteins were obtained.

In this study, to better understand the molecular mechanisms of magnetite biomineralization, we use RNA sequencing (RNA-seq) to construct the transcriptome data set of radular tissue in *C. stelleri*. RNA-seq based transcriptome analysis reveals highly expressed transcripts in both non-mineralized and mineralized teeth regions. Furthermore, the transcriptome data was used to identify the proteins in mineralized teeth.

Results

RNA-seq and *de novo* transcriptome assembly

Radular teeth are formed within the radular tissue. Here, teeth are surrounded by epithelial cells (Fig. 1a) and it is likely that the proteins required for teeth formation may be supplied from these cells. To construct expressed sequence data sets from the radular tissue, RNA was extracted from this tissue (including radular teeth and epithelial cells). After dissection, radular tissue from the non-mineralized teeth region (first 8 to 12 rows of transparent teeth) and those from the mineralized teeth region (following 2 to 5 rows of partially-mineralized reddish-brown colored teeth plus 5 to 6 rows of mineralized magnetic teeth) (Fig. 1a) were separated and RNA was extracted from each sample. RNA-seq was conducted on the extracted RNA. The reads obtained from the non-mineralized teeth region and the mineralized teeth region were then assembled together into 187,980 transcripts. The average length of transcripts is 705 bp and the maximum length is 16,738 bp (with a N50 length of 1,110 bp, Table 1).

Highly expressed transcripts in mineralized and non-mineralized teeth regions

To detect highly expressed transcripts in mineralized and non-mineralized teeth region, each set of reads from those samples was first aligned to the assembled 187,980 transcripts.

Among the twenty most highly expressed transcripts in the non-mineralized teeth region, five transcripts are isoforms of Cs17717|c0_g1, which did not show similarity to any known proteins (Supplementary Table S1). There are two transcripts encoding peritrophin-like proteins: Cs79475|c0_g1_i1 and Cs70642|c0_g2_i1. Peritrophin and peritrophin-like proteins have been found in both insects and crustaceans. These proteins have chitin binding domains and have been thought to be involved in immune defense or food digestion¹⁹. Cs25220|c1_g1_i1 shares a similarity to a calcium carbonate biomineralization related protein, Pif, in mollusks²⁰. Four proteins, including peritrophin-like and Pif-like proteins, described above have a type-2 chitin-binding domain²¹. The transcripts of a cytochrome c oxidase subunit I (Cs76629|c0_g1_i1) and a ferritin subunit 2 (Cs75144|c0_g1_i1) that are involved in iron accumulation were included in highly expressed transcripts in the non-mineralized teeth region. Furthermore, four transcripts encoding radular teeth proteins, which are mentioned below, were also included.

Distinct profiles were observed in the twenty most highly expressed transcripts in the mineralized teeth region (Supplementary Table S2). The mitochondrial respiratory chain enzymes constitute 35% of the highly expressed transcripts. Those enzymes include cytochrome c oxidase subunit I, cytochrome c oxidase subunit II, cytochrome c oxidase subunit III, ATP synthase F0 subunit 6, NADH dehydrogenase subunit 5 and NADH dehydrogenase subunit 1. Those are encoded in mitochondrial genome of *C. stelleri*²². In addition, the chitin binding beak protein 3 (Cs12250|c0_g1_i1), which has been found from squid beaks was included. Five transcripts (25%) are isoforms of Cs22243|c0_g7, whose function is unknown.

Analysis of ferritin genes

Ferritin is an iron transport and storage protein which assembles to form a protein cage containing up to 4500 iron atoms in the form of a hydrous ferric oxide-based mineral. Since ferritins were detected and

isolated from epithelial cells of the radular tissue in *C. stelleri*²³, ferritin has been thought to be involved in iron transport for tooth mineralization. In this study, the transcripts showing homology with ferritin genes were screened from the 134,993 transcripts. As a result, the deduced amino acid sequences of the four transcripts share homology with ferritin protein from the chiton *Liolophura japonica*. Among four ferritin homologs, two are identical except one amino acid substitution (Cs22563|c0_g1_i1 and Cs75144|c0_g1_i1) (Supplementary Fig. S1). The sequence of Cs17042|c0_g1_i1 lacked the 5' terminus of the full-length cDNA. Until now, two types of ferritins, cytoplasmic ferritins and secreted ferritins, have been isolated from mollusks²⁴. The results of a homology search indicated that Cs90734|c0_g1_i1 shared a homology with secreted ferritins, while Cs22563|c0_g1_i1, Cs75144|c0_g1_i1 and Cs17042|c0_g1_i1 are more similar to cytoplasmic ferritins. The phylogenetic analysis was conducted using the sequences of known secreted and cytoplasmic ferritins from Mollusca and Arthropoda. As a result, Cs90734|c0_g1_i1 ferritin clustered with secreted ferritins and the other three ferritins clustered with cytoplasmic ferritins (Supplementary Fig. S2). Evidently, compared to other ferritins in *C. stelleri*, Cs90734|c0_g1_i1 has distinct structural features commonly observed in secreted-type ferritins such as: (1) a ~ 40 amino acid residue insertion located in the middle of the putative loop region, (2) the putative metal binding residues, which are highly conserved in cytoplasmic ferritins, are replaced with other amino acids. However, it does not contain signal sequences that exist in other secreted ferritins. Among four ferritin genes, Cs75144|c0_g1_i1 showed a predominant expression, suggesting its importance in radular teeth formation (Supplementary Table S3). Notably, Cs75144|c0_g1_i1 was more highly expressed in the non-mineralized teeth region than in the mineralized teeth region.

Proteome analysis of radular teeth proteins

In chitons, each radular tooth consists of a mineralized cusp, base (also referred to as stylus) supporting the mineralized cusp and a radular membrane (Fig. 1b). Previously, the mineralized cusps were physically separated from base plus radular membrane and the proteins were extracted from each part of radular teeth using boiling SDS¹⁵. Extracted proteins were digested into peptides and analyzed with nano-LC-

MS. In that work, the lack of available genetic information on *C. stelleri* required a MS/MS ion search against the NCBI nr database and *de novo* peptide sequencing of MS/MS spectra in order to identify the proteins.

In this study, a full six-frame translated database was initially generated from assembled transcripts of *C. stelleri*. Then, the previously obtained MS/MS spectra of teeth proteins were analyzed by a MS/MS ion search using the translated database. Data sets of MS/MS spectra from replicate analyses (n=2) were used for the search. As a result, the peptide sequences from the mineralized cusp fraction were assigned to 232 transcripts (Supplementary Table S4) and those from the base plus membrane fraction were assigned to 114 transcripts (Supplementary Table S5). A high level of redundancy was present in the results as many of the peptides were shared by different proteins. This could be due to the presence of isoforms or artificial redundancy caused by sequencing errors or assembly errors²⁵. The proteins matching the same set of peptides are listed in Supplementary Table S4 and Table S5. From FPKM expression data, the proteins encoded by the transcripts which showed the greatest expression were selected for further analysis. Annotations of the proteins are based on the results of BlastP analyses.

In total, 31 proteins were identified from the base plus membrane fraction (Supplementary Table S5). From this, 22 proteins were identified from 1st analysis and 24 proteins were identified from 2nd analysis. From the cusp fraction, a total of 77 proteins were identified. 61 proteins were identified from the 1st analysis and 54 proteins were identified from the 2nd analysis. The proteins identified from the cusp fraction include four proteins: Myoglobin, actin, elongation factor 1 alpha and Arginine kinase, which are encoded by the top 20 most highly expressed transcripts in the non-mineralized teeth region (Cs77196|c0_g2_i1, Cs47470|c2_g3_i1, Cs24354|c0_g1_i1 and Cs82664|c0_g1_i1; Supplementary Table S1). An iron transporting protein, ferritin, whose gene was also highly expressed in non-mineralized teeth region, was not identified. The 19 proteins were identified from both cusp and base plus membrane fractions. It includes a large number of structural proteins such as actin, filamin-A, tubulin and fibrillin. The chitin-binding beak protein 3 encoded by a highly expressed Cs12250|c0_g1_i1 is also included.

Elongation factor 1 alpha (Cs24354|c0_g1_i1) was identified from blank sample and is thought to be a contaminant.

Mineralized cusp-specific proteins

The mineralized cusp proteins, which were identified in both 1st and 2nd analyses (and not identified from the base plus membrane fraction), were listed as mineralized cusp-specific proteins (Table 2).

Two globin-like proteins were identified as mineralized cusp-specific proteins with the highest MASCOT scores (Cs77196|c0_g2_i1 and Cs77024|c0_g1_i1). The sequences of both proteins contain heme-binding domains of globin proteins. Cs77196|c0_g2_i1 showed the best match with radular muscle myoglobin of *Liolophura japonica*²⁶. This protein is abundant in the cusp region and its N-terminal sequence was determined in our previous study¹⁵. Cs77024|c0_g1_i1 showed a similarity with a neuroglobin-like protein. An acidic peptide sequence detected in the previous study which was not assigned to any protein at that time was the part of Cs77024|c0_g1_i1¹⁵. Sequence alignment with other neuroglobins revealed that the acidic region of Cs77024|c0_g1_i1 was unique to this protein.

Three proteins shared a similarity with peroxidasin. Among them, two were isoforms: Cs18149|c2_g1_i5 and Cs18149|c2_g1_i2. All peroxidasin possess N-terminal signal sequences and heme-dependent peroxidase domains (Supplementary Table S6). Furthermore, a chitin-binding domain was found at the C-terminus of Cs18149|c2_g1_i5. A number of peroxidasin homologues are in the transcriptome data, and although there are 3'- or 5'- truncated forms, only Cs18149|c2_g1_i5 has a chitin-binding domain.

Two proteins correspond to antioxidant enzymes, peroxiredoxin 6 (Cs59318|c0_g1_i1) and superoxide dismutase (Cu-Zn) (Cs69741|c0_g1_i1). As a result of subcellular localization analysis, peroxiredoxin 6 was predicted as a cytoplasmic protein, while superoxide dismutase (Cu-Zn) was predicted as an extracellular protein (Supplementary Table S6).

Two proteins were homologous to the metal transport proteins, such as a copper transport protein ATOX1-like (Cs22616|c0_g1_i1) and a hephaestin-like protein (Cs46312|c2_g1_i1). Hephaestin

is a protein that possesses ferroxidase activity and is believed to mediate intestinal iron absorption in mammals^{27,28}. Both membrane-associated and soluble forms of hephaestin have been reported^{29,30}. A domain search predicted the presence of a chitin-binding domain in hephaestin-like protein (Cs46312|c2_g1_i1). It has three predicted β -sheet structures and several aromatic amino acids that are characteristic to a type-3 chitin-binding domain³¹. Cs22616|c0_g1_i1 has a heavy metal-associated domain of ATOX1, which contain two cysteine residues for metal ion binding. ATOX1 is a copper chaperone that plays a role in incorporating Cu into the copper-dependent enzymes such as hephaestin³².

Other proteins include Arginine kinase (Cs82664|c0_g1_i1), hillarin (Cs28725|c1_g1_i1) and cytosolic malate dehydrogenase (Cs28687|c2_g1_i1), which are predicted to localize in the mitochondria (Supplementary Table S6). Furthermore, nuclear proteins (Eukaryotic initiation factor 4A-II, partial and histone H2B 8-like) and cytoskeletal proteins (muscle-specific protein 20-like isoform X2 and filamin-A-like isoforms) were identified. Protein usf-like (Cs75674|c0_g4_i1) that has a dienelactone hydrolase domain was identified with a high MASCOT score and a 33% sequence coverage.

There is only one protein (Cs68435|c0_g1_i1) that shared no sequence similarity with any known proteins. This protein possesses a unique repetitive sequence and an unusually high proportion of glycine and serine residues. Further analysis was conducted on this novel protein. Since the 5' terminus of the Cs68435|c0_g1_i1 coding sequence was truncated, its full-length sequence was determined by 5'-RACE. The overall sequence consists of a glycine- and serine-rich repetitive region, followed by a tryptophan- and phenylalanine-rich region and a histidine-rich region (Fig. 2). Structure prediction indicates that a disordered structure is present in a glycine- and serine-rich region (Supplementary Fig.S4). A sequence alignment of a WF-rich region in RTMP1 with the chitin-binding domains of four bacterial chitinases indicated that three of five putative chitin interacting residues in the chitin-binding domains were also conserved in RTMP1 (Supplementary Fig. S5)³¹. Three β -strands were predicted in the corresponding region of RTMP1. Based on results of *in silico* analyses, a number of serine residues of Cs68435|c0_g1_i1 are predicted to be phosphorylated. Analyses by several different prediction software

indicated that Cs68435|c0_g1_i1 contains an N-terminal signal sequence (Fig. 2, Supplementary Table S6). This novel protein is termed RTMP1 (radular teeth matrix protein 1).

Gene expression profiles of mineralized cusp-specific proteins

The expression profiles of the transcripts encoding mineralized cusp-specific proteins in different mineralization stages were analyzed based on FPKM values. Expression levels of several transcripts (e.g., Neuroglobin-like, peroxiredoxin 6 and superoxide dismutase (Cu-Zn)) differed in abundance by less than 4-fold between the mineralized teeth and non-mineralized teeth regions (Supplementary Fig. S3). On the other hand, the transcripts whose expression increased more than 4-fold in the non-mineralized teeth region (e.g., protein usf-like, Arginine kinase, Myoglobin and hephaestin-like protein) or mineralized teeth region (e.g., RTMP1, Peroxidase and copper transport protein ATOX1-like) are thought to be differentially expressed during teeth mineralization (Supplementary Fig. S3).

Discussion

In this study, a comprehensive transcriptome data from the radular tissue of *C. stelleri* has been developed for the first time. The constructed transcriptome data provides new insights into the genes highly expressed in radular tissue during teeth formation in *C. stelleri*. Furthermore, by using the transcriptome data, a proteome profile of mineralized cusp-specific proteins were determined. Those transcriptome and proteome data represent invaluable resources for the investigation of the magnetite biomineralization (including iron oxide nucleation, growth and phase transformation) in chitons.

Four homologues of ferritin were identified from the transcriptome data. Among the four ferritin homologs, the cytoplasmic ferritin, Cs75144|c0_g1_i1 is highly expressed in radular tissue. Previously, it was demonstrated that ferritin-containing granules first appear in the epithelial cells located 1-3 rows anterior to the tooth in which iron oxide is first deposited. The amount of granules increases greatly over a few rows, and then gradually decreases¹⁴. Those observations were correlated with the expression pattern of cytoplasmic ferritins (Supplementary Table S3). Since the iron transport is the earliest event in the

tooth formation process, the expression of the ferritin genes might be strongly induced in the non-mineralized teeth region prior to mineralization.

Contrary to the above data, previous research using other chiton species suggested that secreted ferritins accumulated in the epithelial cells of radular tissue during teeth mineralization^{14,33}. In order to determine which ferritin plays a role in teeth mineralization, further studies including protein analysis of epithelial cells are needed.

Although the ferritin gene, Cs75144|c0_g1_i1 was included in the twenty most highly expressed transcripts in the non-mineralized teeth region (Supplementary Table S1), ferritin proteins were not identified from mineralized cusps. This result was consistent with the previous observation that no ferritin granules were detected inside the teeth³⁴. Based on these results, it is likely that iron in ferritin is released and transported inside the teeth in a soluble form, and re-deposited as ferrihydrite. Shaw *et al.* reported the existence of ferritin like granules in the cells located inside of the base (also referred to as stylus)³⁵. However, in this study, we did not detect ferritin from the base plus membrane fraction (Supplementary Table S5). It is possible that sample preparation of the base plus membrane fraction that included repeated wash with 70% ethanol and water¹⁵, could result in the loss of ferritin proteins.

The mitochondrial respiratory chain enzymes were overrepresented among the top-20 most highly expressed transcripts in the mineralized teeth region (Supplementary Table S2). Electron microscopic analysis of partially mineralized teeth have previously shown the presence of numerous mitochondria in the cells surrounding the partially mineralized teeth^{14,36}. It has been suggested that a large amount of energy required for iron transport or transformation of ferrihydrite to magnetite may be provided by those mitochondria^{14,36}.

Proteomic analysis using the *de novo* constructed transcriptome data of radular tissue in *C. stelleri* led to the identification of three-fold more mineralized cusp-specific proteins (22 in total) than our previous report¹⁵. Furthermore, a novel protein that could not be detected by the previous method was identified in this study.

Cytoskeletal proteins (e.g., filamin-A-like isoforms, muscle-specific protein 20-like isoform X2) or nuclear proteins (e.g., Eukaryotic initiation factor 4A-II, partial, histone H2B 8-like), which were apparently unrelated to teeth formation, were identified as mineralized cusp-specific proteins (Table 2). It is likely that those highly expressed proteins were occluded in the teeth during mineralization and identified as mineralized cusp proteins.

Two globin-like proteins were identified as mineralized cusp-specific proteins. Significant amounts of myoglobin, which help supply oxygen to muscle³⁸, have been known to exist in the radular muscles of chiton^{26,39,40}. Therefore, it is likely that the highly expressed myoglobin could contaminate the mineralized cusp fraction. Conversely, as the oxygen concentration strongly affects the redox state of iron, iron release from ferritin and magnetite formation, those proteins could have essential roles for teeth formation and mineral phase transformation. Although the function of the neuroglobin-like protein, Cs77024|c0_g1_i1, is unknown, it has been suggested to play a role in O₂ control as myoglobin⁴¹. Of note, the neuroglobin-like protein, Cs77024|c0_g1_i1, has a unique acidic region that was not present in other neuroglobin-like proteins. The acidic domain of the magnetite morphology-modulating protein, Mms6 has been shown to be essential for its function⁴². Therefore, Cs77024|c0_g1_i1, which has a unique acidic amino acid rich region, might have a specialized function for magnetite formation.

3 out of 22 mineralized cusp-specific proteins (Cs18149|c2_g1_i5, Cs18149|c2_g1_i2 and Cs71263|c1_g1_i1) showed similarities to peroxidasin. Peroxidasin is known to have a hybrid structure consisting of a peroxidase domain and extracellular matrix motifs⁴³. It is secreted extracellularly and mediates the cross-link formation in collagen for tissue development⁴⁴. Notably, among all peroxidasin-like proteins in transcriptome data, only Cs18149|c2_g1_i5 has a chitin binding domain in its C-terminus. It is possible that Cs18149|c2_g1_i5 is secreted extracellularly to bind chitin and catalyze the chemical bond formation to consolidate a scaffold for biomineralization. Interestingly, peroxidasin has been identified from other calcium-based biominerals, including pearl oyster, and has been suggested to be important for biomineral formation⁴⁵⁻⁴⁷.

The biomineralization protein Silicatein, which was isolated from sponge spicules, has been known to catalyze the hydrolysis and polycondensation of silicon alkoxide to form silica^{48,49}. We speculate that the usf-like protein (Cs75674|c0_g4_i1), containing a diene lactone hydrolase domain, could also have a catalytic role contributing to hydrolysis of iron oxide precursors to form magnetite⁵⁰.

Two antioxidant proteins, peroxiredoxin 6 (Cs59318|c0_g1_i1) and superoxide dismutase (Cu-Zn) (Cs69741|c0_g1_i1) were predicted to be localized both inside and outside of the cells (Supplementary Table S6). Since the proposed models of iron release pathways from ferritins involved superoxide radicals⁵¹, superoxide dismutase (Cu-Zn) (Cs69741|c0_g1_i1) could control excess amounts of superoxide radicals. As we mentioned in our previous work, peroxiredoxin 6 (Cs59318|c0_g1_i1) may function to control the reduction of iron species and therefore control the initial phase transformation from ferrihydrite to magnetite by adsorption of Fe²⁺ ions with subsequent solid-state transformation¹⁶.

A hephaestin-like protein (Cs46312|c2_g1_i1) is a ferroxidase protein and may be involved in ferrous iron oxidation into ferric iron. □Of note, hephaestin-like protein contains an N-terminal signal sequence (Supplementary Table S6) and a chitin-binding domain. It suggests that extracellularly secreted hephaestin-like proteins may bind with chitin and catalyze ferrous iron oxidation.

A novel mineralized cusp-specific protein, RTMP1, has a distinctive glycine and serine-rich region (Fig. 2). Similar glycine- and serine-rich domains were found from other biomineral-associated proteins⁵²⁻⁵⁴. The secondary structure of a glycine and serine-rich region was predicted to be disordered and thus might form a flexible structure like the aforementioned biomineral proteins (Supplementary Fig.S4). It is possible that the extended conformation of RTMP1 might provide more mineral binding sites than tightly packed globular proteins⁵⁵. The WF-rich region in RTMP1 resembles a type-3 chitin binding domain of bacterial chitinase, which have β -sheets as well as aromatic and hydrophobic-rich sequences (Supplementary Fig. S5). Thus, similar to those bacterial chitinases, it is likely that RTMP1 binds to crystalline forms of chitin³¹. A number of phosphorylated serines were inferred to reside in RTMP1. Of note, amino acid analysis of matrix proteins in the radula teeth of the chiton *Acanthopleura hirtosa* indicated that there were appreciable amounts of phosphorylated serines⁵⁶. In other biominerals,

phosphorylation is often required for mineral-protein interaction^{57,58}. These results support the idea that the extracellularly secreted RTMP1, which is likely to be phosphorylated, may interact with chitin and promote iron oxide nucleation on chitin fibers.

Expression analysis of the transcripts encoding the mineralized cusp-specific proteins provides additional insight into the functions of those proteins for teeth mineralization. Except for the aforementioned possible contaminants, the transcripts highly expressed throughout teeth mineralization (e.g., Neuroglobin-like, peroxiredoxin 6 and superoxide dismutase (Cu-Zn)) may be important for the entire process of mineralization (Supplementary Fig. S3). Conversely, the transcripts highly expressed in the non-mineralized teeth region (e.g., protein usf-like, Arginine kinase, hephaestin-like protein) may play a role in the former processes, including initial iron transport (Supplementary Fig. S3). The transcripts highly expressed in the mineralized teeth region (e.g., RTMP1, Peroxidasins, copper transport protein ATOX1-like) may be involved in latter processes, such as ferrihydrite nucleation, growth, and phase transformation to magnetite (Supplementary Fig. S3).

By analyzing gene deletion mutants, several proteins important for magnetite mineralization in magnetotactic bacteria have been reported⁶⁻⁹. Those proteins include redox-active proteins that may control oxidation and reduction of iron for magnetite mineralization^{59,60}, magnetotactic bacteria-specific proteins related to maturation of magnetite crystals^{6,7}, cation diffusion facilitators⁶¹ and proteins with protease domains⁶². When compared to the magnetotactic bacterial proteins, redox-active proteins (peroxiredoxin-6, superoxide dismutase (Cu-Zn) and hephaestin-like protein) were also identified from *C. stelleri* as mineralized cusp-specific proteins. These proteins may play roles in oxidation and reduction of iron for transport, nucleation and magnetite mineralization.

Conclusions

In this study, we constructed the first comprehensive transcriptome data sets from radular tissue in the gumboot chiton, *Cryptochiton stelleri*. Transcript expression analysis revealed that the most expressed transcripts in the non-mineralized teeth region include ferritin genes, while those in the mineralized teeth

region include genes of a number of mitochondrial respiratory chain enzymes. These results concur with the previous microscopic observation of developing radular teeth in chitons and suggest the importance of these genes in teeth mineralization. Furthermore, using proteomic analysis, 22 mineralized cusp specific proteins including a novel protein were identified. These proteins are good candidates for future research of iron oxide mineralization in chitons. The results obtained from this study are valuable in terms of providing the first overview of radular teeth formation.

Methods

RNA isolation and cDNA library construction

Live specimens of *C. stelleri* were collected and maintained as previously described¹⁶. Collection of *Cryptochiton stelleri* is permitted by the Department of Fish and Game and the California Natural Resources Agency. Radular tissues were isolated from freshly dissected *C. stelleri*. After radular tissue isolation, non-mineralized teeth region (first 8 to 12 rows of transparent teeth) and mineralized teeth region (following 2 to 5 rows of partially-mineralized reddish-brown colored teeth plus 5 to 6 rows of mineralized magnetic teeth) were separated from whole radulae and frozen immediately in liquid nitrogen and stored at -80°C until used. Total RNA was extracted from each radular tissue region separately using TRI reagent (Molecular Research Center), and purified using RNeasy mini kit (QIAGEN) following the manufacturer's instructions. Integrity and quantity of extracted RNA was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA Libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit (Illumina).

Sequencing and *de novo* transcriptome assembly

The constructed libraries were sequenced on an Illumina Hiseq 2500 platform and paired-end reads were generated. From raw reads, adapter sequences were removed using cutadapt⁶³. Reads were then quality trimmed using Trimmomatic⁶⁴. The trimmed reads from two samples (non-mineralized teeth region and

mineralized teeth region) were pooled and assembled *de novo* into a transcriptome reference using Trinity⁶⁵.

Bioinformatic analysis

The trimmed reads obtained from two samples (non-mineralized teeth region and mineralized teeth region) were aligned to the above constructed transcriptome reference using Bowtie¹⁷. Based on the mapping results, the expression levels of transcripts were estimated by fragments per kilobase of transcript per million fragments mapped value (FPKM) using RSEM¹⁸. A homology search was conducted using the BLAST program⁶⁶ against NCBI nr database for functional annotation of transcripts and proteins. Homologues of ferritin genes were searched from the transcriptome data of *C. stelleri* using local BLAST. The conserved domains of protein sequences were searched with SMART⁶⁷. MEGA version 6⁶⁸ was used for phylogenetic analysis of ferritin proteins with the maximum likelihood (ML) method. The following ferritin proteins from Mollusca and Arthropoda were used: LjFer (*Liolophura japonica*, BAA21810), MmxFer (*Meretrix meretrix*, AAZ20754), ScFer (*Sinonovacula constricta*, ACZ65230), AiFer2 (*Argopecten irradians*, AEN71561), AiFer3 (*Argopecten irradians*, AEN71564), PyFer1 (*Patinopecten yessoensis*, AGK92812), PyFer2 (*Patinopecten yessoensis*, AGK92813), PyFer4 (*Patinopecten yessoensis*, AGK92815), LsFer (*Lymnaea stagnalis*, CAA40097), CrFer-H1 (*Carcinoscorpius rotundicauda*, AAW22505), CrFer-H2 (*Carcinoscorpius rotundicauda*, AAW22506), HddFer (*Haliothis discus discus*, ABG88845), EsFer1 (*Eriocheir sinensis*, ADF87490), EsFer2 (*Eriocheir sinensis*, ADF87491), PyFerS1 (*Patinopecten yessoensis*, AHH31562), PyFerS2 (*Patinopecten yessoensis*, AHH31563), SdFer (*Suberites domuncula*, CAC84556). The analysis was run with the Jones-Taylor-Thornton (JTT) model. Bootstrap trials were replicated 1000 times. The putative phosphorylation sites of RTMP1 was predicted in silico using NetPhos 3.1 Server (<http://www.cbs.dtu.dk/services/NetPhos/>)⁶⁹. The potential phosphorylation sites with scores higher than 0.9 were considered to be positive. Disordered regions in RTMP1 were predicted using the DISOPRED server⁷⁰. Jpred⁷¹ was used to predict the secondary structures of the WF-rich region in RTMP1.

Subcellular localization of identified proteins were predicted using the following software: SignalP 4.1 ⁷², TargetP1.1 ⁷³, WOLF PSORT ⁷⁴, MultiLoc2 ⁷⁵ and Signal-BLAST ⁷⁶. Amino acid sequence alignments were conducted using Clustal Omega software⁷⁷.

Proteomic analysis

A detailed method of protein extraction from the radular teeth of *C. stelleri* and following LC-MS/MS analysis were described in our previous work ¹⁵. In this study, first, a six frame translated database was created from a transcriptome data of *C. stelleri*. Then the previously obtained MS/MS spectra of radular teeth proteins were searched against a six-frame translated database with the MASCOT algorithm for protein identification. The parameters used for MASCOT were same as previously described ¹⁵. The protein identification was based on two technical replicates.

5' Rapid amplification of cDNA ends (RACE)

5' rapid amplification of cDNA ends (RACE) was performed with 5'-Full RACE Core Set (Takara) to determine the full length sequences of Cs68435|c0_g1_i1 and Cs59318|c0_g1_i1. Total RNA was extracted from radular tissue as described above. 5'-RACE products were cloned into pMD20-T vector (Takara) and sequenced.

Data availability

The reads derived from Illumina Hiseq of the analyzed samples have been deposited in DDBJ Sequence Read Archive with accession number DRA007106.

References

- 1 Lowenstam, H. A. Magnetite in Dentine Capping in Recent Chitons. *Geological Society of America Bulletin* **73**, 435-438 (1962).
- 2 Blakemore, R. Magnetotactic bacteria. *Science* **190**, 377-379 (1975).

- 3 Gould, J. L., Kirschvink, J. L. & Deffeyes, K. S. Bees have magnetic remanence. *Science* **201**, 1026-1028, doi:10.1126/science.201.4360.1026 (1978).
- 4 Kirschvink, J. L., Walker, M. M., Chang, S.-B., Dizon, A. E. & Peterson, K. A. Chains of single-domain magnetite particles in chinook salmon, *Oncorhynchus tshawytscha*. *J Comp Physiol A* **157**, 375-381 (1985).
- 5 Walcott, C., Gould, J. L. & Kirschvink, J. L. Pigeons have magnets. *Science* **205**, 1027-1029 (1979).
- 6 Tanaka, M., Mazuyama, E., Arakaki, A. & Matsunaga, T. MMS6 Protein Regulates Crystal Morphology during Nano-sized Magnetite Biomineralization in Vivo. *Journal of Biological Chemistry* **286**, 6386-6392, doi:10.1074/jbc.M110.183434 (2011).
- 7 Arakaki, A., Yamagishi, A., Fukuyo, A., Tanaka, M. & Matsunaga, T. Co-ordinated functions of Mms proteins define the surface structure of cubo-octahedral magnetite crystals in magnetotactic bacteria. *Molecular Microbiology* **93**, 554-567, doi:10.1111/mmi.12683 (2014).
- 8 Murat, D., Quinlan, A., Vali, H. & Komeili, A. Comprehensive genetic dissection of the magnetosome gene island reveals the step-wise assembly of a prokaryotic organelle. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 5593-5598, doi:10.1073/pnas.0914439107 (2010).
- 9 Lohsse, A. *et al.* Genetic Dissection of the mamAB and mms6 Operons Reveals a Gene Set Essential for Magnetosome Biogenesis in *Magnetospirillum gryphiswaldense*. *Journal of Bacteriology* **196**, 2658-2669, doi:10.1128/jb.01716-14 (2014).
- 10 Weaver, J. C. *et al.* Analysis of an ultra hard magnetic biomineral in chiton radular teeth. *materialstoday* **13**, 42-52 (2010).
- 11 Grunenfelter, L. K. *et al.* Stress and Damage Mitigation from Oriented Nanostructures within the Radular Teeth of *Cryptochiton stelleri*. *Advanced Functional Materials* **24**, 6093-6104, doi:10.1002/adfm.201401091 (2014).
- 12 de Obaldia, E. E., Jeong, C., Grunenfelter, L. K., Kisailus, D. & Zavattieri, P. Analysis of the mechanical response of biomimetic materials with highly oriented microstructures through 3D

- printing, mechanical testing and modeling. *Journal of the Mechanical Behavior of Biomedical Materials* **48**, 70-85, doi:10.1016/j.jmbbm.2015.03.026 (2015).
- 13 de Obaldia, E. E., Herrera, S., Grunenfelder, L. K., Kisailus, D. & Zavattieri, P. Competing mechanisms in the wear resistance behavior of biomineralized rod-like microstructures. *Journal of the Mechanics and Physics of Solids* **96**, 511-534, doi:10.1016/j.jmps.2016.08.001 (2016).
 - 14 Nesson, M. H. & Lowenstam, H. A. in *Magnetite Biomineralization and Magnetoreception in Organisms* (eds J. L. Kirschvink, D. S. Jones, & B. J. MacFadden) Ch. 16, 333-363 (Springer US, 1985).
 - 15 Nemoto, M. *et al.* Proteomic analysis from the mineralized radular teeth of the giant Pacific chiton, *Cryptochiton stelleri* (Mollusca). *Proteomics* **12**, 2890-2894, doi:10.1002/pmic.201100473 (2012).
 - 16 Wang, Q. Q. *et al.* Phase Transformations and Structural Developments in the Radular Teeth of *Cryptochiton Stelleri*. *Advanced Functional Materials* **23**, 2908-2917, doi:10.1002/adfm.201202894 (2013).
 - 17 Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* **10**, doi:10.1186/gb-2009-10-3-r25 (2009).
 - 18 Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *Bmc Bioinformatics* **12**, doi:10.1186/1471-2105-12-323 (2011).
 - 19 Tellam, R. L., Wijffels, G. & Willadsen, P. Peritrophic matrix proteins. *Insect Biochemistry and Molecular Biology* **29**, 87-101, doi:10.1016/s0965-1748(98)00123-4 (1999).
 - 20 Suzuki, M. *et al.* An Acidic Matrix Protein, Pif, Is a Key Macromolecule for Nacre Formation. *Science* **325**, 1388-1390, doi:10.1126/science.1173793 (2009).
 - 21 Elvin, C. M. *et al.* Characterization of a major peritrophic membrane protein, peritrophin-44, from the larvae of *Lucilia cuprina* - cDNA and deduced amino acid sequences. *Journal of Biological Chemistry* **271**, 8925-8935, doi:10.1074/jbc.271.15.8925 (1996).
 - 22 Irisarri, I., Eernisse, D. J. & Zardoya, R. Molecular phylogeny of Acanthochitonina (Mollusca:

- Polyplacophora: Chitonida): three new mitochondrial genomes, rearranged gene orders and systematics. *Journal of Natural History* **48**, 2825-2853, doi:10.1080/00222933.2014.963721 (2014).
- 23 Towe, K. M., Lowenstam, H. A. & Nesson, M. H. Invertebrate ferritin: Occurrence in Mollusca. *Science* **142**, 63-64 (1963).
 - 24 Vondarl, M., Harrison, P. M. & Bottke, W. cDNA cloning and deduced amino acid sequence of two ferritins: soma ferritin and yolk ferritin, from the snail *Lymnaea stagnalis* L. *European Journal of Biochemistry* **222**, 353-366 (1994).
 - 25 Nesvizhskii, A. I. & Aebersold, R. Interpretation of shotgun proteomic data - The protein inference problem. *Molecular & Cellular Proteomics* **4**, 1419-1440, doi:10.1074/mcp.R500012-MCP200 (2005).
 - 26 Suzuki, T., Furukohri, T. & Okamoto, S. Amino acid sequence of myoglobin from the chiton *Liolophura japonica* and a phylogenetic tree for molluscan globins. *Journal of Protein Chemistry* **12**, 45-50, doi:10.1007/bf01024913 (1993).
 - 27 Fuqua, B. K. *et al.* The Multicopper Ferroxidase Hephaestin Enhances Intestinal Iron Absorption in Mice. *Plos One* **9**, doi:10.1371/journal.pone.0098792 (2014).
 - 28 Vulpe, C. D. *et al.* Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nature Genetics* **21**, 195-199 (1999).
 - 29 Ranganathan, P. N., Lu, Y., Fuqua, B. K. & Collins, J. F. Immunoreactive Hephaestin and ferroxidase activity are present in the cytosolic fraction of rat enterocytes. *Biometals* **25**, 687-695, doi:10.1007/s10534-012-9527-9 (2012).
 - 30 Martins, R., Silva, B., Proenca, D. & Faustino, P. Differential HFE Gene Expression Is Regulated by Alternative Splicing in Human Tissues. *Plos One* **6**, doi:10.1371/journal.pone.0017542 (2011).
 - 31 Ikegami, T. *et al.* Solution structure of the chitin-binding domain of *Bacillus circulans* WL-12 chitinase A1. *Journal of Biological Chemistry* **275**, 13654-13661, doi:10.1074/jbc.275.18.13654 (2000).

- 32 Pufahl, R. A. *et al.* Metal ion chaperone function of the soluble Cu(I) receptor Atx1. *Science* **278**, 853-856, doi:10.1126/science.278.5339.853 (1997).
- 33 Okoshi, K. & Hamaguchi, M. in *KAIYOUSEIBUTU NO KINOU (Function of marine organism)* (ed Yoshio Takei) 345-356 (Tokai Daigaku Shuppan, 2005).
- 34 Lowenstam, H. A. Minerals formed by organisms. *Science* **211**, 1126-1131 (1981).
- 35 Shaw, J. A. *et al.* The Chiton Stylus Canal: An Element Delivery Pathway for Tooth Cusp Biomineralization. *Journal of Morphology* **270**, 588-600, doi:10.1002/jmor.10705 (2009).
- 36 Shaw, J. A. *et al.* Ultrastructure of the Epithelial Cells Associated with Tooth Biomineralization in the Chiton *Acanthopleura hirtosa*. *Microscopy and Microanalysis* **15**, 154-165, doi:10.1017/s1431927609090230 (2009).
- 37 Tan, Y. P. *et al.* Infiltration of chitin by protein coacervates defines the squid beak mechanical gradient. *Nature Chemical Biology* **11**, 488-+, doi:10.1038/nchembio.1833 (2015).
- 38 Suzuki, T. & Imai, K. Evolution of myoglobin. *Cellular and Molecular Life Sciences* **54**, 979-1004, doi:10.1007/s000180050227 (1998).
- 39 Terwilliger, R. C. & Read, K. R. H. The radular muscle myoglobins of the amphineuran mollusc, *Acanthopleura granulata* Gmelin. *Comparative Biochemistry and Physiology* **29**, 551-560 (1969).
- 40 Terwilliger, R. C. & Read, K. R. H. The radular muscle myoglobins of the amphineuran molluscs, *Katharina tunicata* Wood, *Cryptochiton stelleri* Middendorf, and *Mopalia muscosa* Gould. *International Journal of Biochemistry* **1**, 281-291 (1970).
- 41 Burmester, T. & Hankeln, T. What is the function of neuroglobin? *Journal of Experimental Biology* **212**, 1423-1428, doi:10.1242/jeb.000729 (2009).
- 42 Yamagishi, A., Narumiya, K., Tanaka, M., Matsunaga, T. & Arakaki, A. Core Amino Acid Residues in the Morphology-Regulating Protein, Mms6, for Intracellular Magnetite Biomineralization. *Scientific Reports* **6**, doi:10.1038/srep35670 (2016).
- 43 Nelson, R. E. *et al.* Peroxidase: a novel enzyme-matrix protein of *Drosophila* development. *Embo Journal* **13**, 3438-3447 (1994).

- 44 Bhave, G. *et al.* Peroxidasin forms sulfilimine chemical bonds using hypohalous acids in tissue genesis. *Nature Chemical Biology* **8**, 784-790, doi:10.1038/nchembio.1038 (2012).
- 45 Liu, C. *et al.* In-depth proteomic analysis of shell matrix proteins of *Pinctada fucata*. *Scientific Reports* **5**, doi:10.1038/srep17269 (2015).
- 46 Germer, J., Mann, K., Worheide, G. & Jackson, D. J. The Skeleton Forming Proteome of an Early Branching Metazoan: A Molecular Survey of the Biomineralization Components Employed by the Coralline Sponge *Vaceletia* Sp. *Plos One* **10**, doi:10.1371/journal.pone.0140100 (2015).
- 47 Luo, Y. J. *et al.* The *Lingula* genome provides insights into brachiopod evolution and the origin of phosphate biomineralization. *Nature Communications* **6**, doi:10.1038/ncomms9301 (2015).
- 48 Cha, J. N. *et al.* Silicatein filaments and subunits from a marine sponge direct the polymerization of silica and silicones in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 361-365, doi:10.1073/pnas.96.2.361 (1999).
- 49 Shimizu, K., Cha, J., Stucky, G. D. & Morse, D. E. Silicatein alpha: Cathepsin L-like protein in sponge biosilica. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 6234-6238, doi:10.1073/pnas.95.11.6234 (1998).
- 50 Bharde, A. *et al.* Extracellular biosynthesis of magnetite using fungi. *Small* **2**, 135-141, doi:10.1002/sml.2005000180 (2006).
- 51 Melman, G. *et al.* Iron release from ferritin by flavin nucleotides. *Biochimica Et Biophysica Acta-General Subjects* **1830**, 4669-4674, doi:10.1016/j.bbagen.2013.05.031 (2013).
- 52 Sarashina, I. & Endo, K. The complete primary structure of molluscan shell protein 1 (MSP-1), an acidic glycoprotein in the shell matrix of the scallop *Patinopecten yessoensis*. *Marine Biotechnology* **3**, 362-369, doi:10.1007/s10126-001-0013-6 (2001).
- 53 Shen, X., Belcher, A. M., Hansma, P. K., Stucky, G. D. & Morse, D. E. Molecular cloning and characterization of lustrin A, a matrix protein from shell and pearl nacre of *Haliotis rufescens*. *J Biol Chem* **272**, 32472-32481 (1997).
- 54 Kroger, N., Deutzmann, R. & Sumper, M. Polycationic peptides from diatom biosilica that direct

- silica nanosphere formation. *Science* **286**, 1129-1132, doi:10.1126/science.286.5442.1129 (1999).
- 55 Magdalena, W., Piotr, D. & O., A. in *Advanced Topics in Biomineralization* (ed Jong Seto) (2012).
- 56 Evans, L. A., Macey, D. J. & Webb, J. Distribution and composition of matrix protein in the radula teeth of the chiton *Acanthopleura hirtosa*. *Marine Biology* **109**, 281-286, doi:10.1007/bf01319396 (1991).
- 57 Kroger, N., Lorenz, S., Brunner, E. & Sumper, M. Self-assembly of highly phosphorylated silaffins and their function in biosilica morphogenesis. *Science* **298**, 584-586, doi:10.1126/science.1076221 (2002).
- 58 Kwak, S. Y. *et al.* Role of 20-kDa Amelogenin (P148) Phosphorylation in Calcium Phosphate Formation in Vitro. *Journal of Biological Chemistry* **284**, 18972-18979, doi:10.1074/jbc.M109.020370 (2009).
- 59 Raschdorf, O., Muller, F. D., Posfai, M., Plitzko, J. M. & Schuler, D. The magnetosome proteins MamX, MamZ and MamH are involved in redox control of magnetite biomineralization in *Magnetospirillum gryphiswaldense*. *Molecular Microbiology* **89**, 872-886, doi:10.1111/mmi.12317 (2013).
- 60 Jones, S. R. *et al.* Genetic and biochemical investigations of the role of MamP in redox control of iron biomineralization in *Magnetospirillum magneticum*. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 3904-3909, doi:10.1073/pnas.1417614112 (2015).
- 61 Uebe, R. *et al.* The cation diffusion facilitator proteins MamB and MamM of *Magnetospirillum gryphiswaldense* have distinct and complex functions, and are involved in magnetite biomineralization and magnetosome membrane assembly. *Molecular Microbiology* **82**, 818-835, doi:10.1111/j.1365-2958.2011.07863.x (2011).
- 62 Yang, W. *et al.* mamO and mamE genes are essential for magnetosome crystal biomineralization in *Magnetospirillum gryphiswaldense* MSR-1. *Research in Microbiology* **161**, 701-705, doi:10.1016/J.resmic.2010.07.002 (2010).
- 63 Martin, M. CUTADAPT removes adapter sequences from high-throughput sequencing reads.

- EMBnet.journal* **17**, 10-12, doi:10.14806/ej.17.1.200 (2011).
- 64 Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114-2120, doi:10.1093/bioinformatics/btu170 (2014).
- 65 Haas, B. J. *et al.* De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nature Protocols* **8**, 1494-1512, doi:10.1038/nprot.2013.084 (2013).
- 66 Altschul, S. F. *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389-3402, doi:10.1093/nar/25.17.3389 (1997).
- 67 Schultz, J., Milpetz, F., Bork, P. & Ponting, C. P. SMART, a simple modular architecture research tool: Identification of signaling domains. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 5857-5864, doi:10.1073/pnas.95.11.5857 (1998).
- 68 Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* **30**, 2725-2729, doi:10.1093/molbev/mst197 (2013).
- 69 Blom, N., Gammeltoft, S. & Brunak, S. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *Journal of Molecular Biology* **294**, 1351-1362, doi:10.1006/jmbi.1999.3310 (1999).
- 70 Ward, J. J., McGuffin, L. J., Bryson, K., Buxton, B. F. & Jones, D. T. The DISOPRED server for the prediction of protein disorder. *Bioinformatics* **20**, 2138-2139, doi:10.1093/bioinformatics/bth195 (2004).
- 71 Cuff, J. A., Clamp, M. E., Siddiqui, A. S., Finlay, M. & Barton, G. J. JPred: a consensus secondary structure prediction server. *Bioinformatics* **14**, 892-893, doi:10.1093/bioinformatics/14.10.892 (1998).
- 72 Petersen, T. N., Brunak, S., von Heijne, G. & Nielsen, H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods* **8**, 785-786, doi:10.1038/nmeth.1701 (2011).
- 73 Emanuelsson, O., Nielsen, H., Brunak, S. & von Heijne, G. Predicting subcellular localization of

- proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology* **300**, 1005-1016, doi:10.1006/jmbi.2000.3903 (2000).
- 74 Horton, P. *et al.* WoLF PSORT: protein localization predictor. *Nucleic Acids Research* **35**, W585-W587, doi:10.1093/nar/gkm259 (2007).
- 75 Blum, T., Briesemeister, S. & Kohlbacher, O. MultiLoc2: integrating phylogeny and Gene Ontology terms improves subcellular protein localization prediction. *Bmc Bioinformatics* **10**, doi:10.1186/1471-2105-10-274 (2009).
- 76 Frank, K. & Sippl, M. J. High-performance signal peptide prediction based on sequence alignment techniques. *Bioinformatics* **24**, 2172-2176, doi:10.1093/bioinformatics/btn422 (2008).
- 77 Sievers, F. *et al.* Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology* **7**, doi:10.1038/msb.2011.75 (2011).

Acknowledgements

This work was supported by Program to Disseminate Tenure Tracking System from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (to M.N.), Kato Memorial Bioscience Foundation (to M.N.) and ARO: W911NF-15-1-0306 (to D.K.).

Author Contributions

M.N. and D.K. designed the research; M.N., D.R., S.H. and S.P. performed the experiments; M.N., T.T., K.I. and D.K. analyzed the data; M.N. and D.K. wrote the paper.

Additional Information

Competing Interests: The authors declare no competing interests.

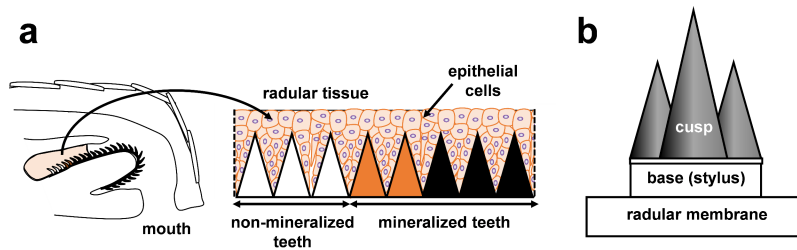


Figure 1. Schematics of radular tissues used in this study. **a**, radular tissues of the giant pacific chiton, *Cryptochiton stelleri*. **b**, individual radular teeth, which are composed of mineralized cusp, base and radular membrane.

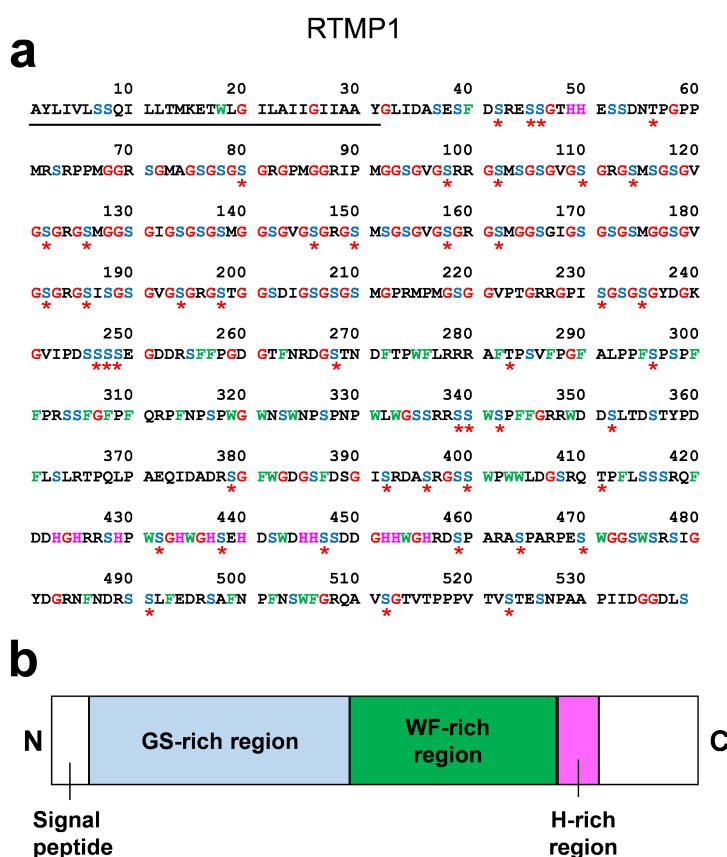


Figure 2. Amino acid sequence of a novel mineralized cusp-specific protein, radular teeth matrix protein 1 (RTMP1). **a**, The entire amino acid sequence of RTMP1 deduced from the cDNA sequence. It consists of 539 amino acids and abundant in glycine (red) and serine (cyan). The underline indicates a putative signal sequence. The putative phosphorylation sites are indicated by asterisks. **b**, Schematic of

the domain structure of RTMP1. GS-rich repetitive region, WF-rich, and H-rich regions are represented by pale blue, green, and pink boxes.

Table 1. Summary of *C. stelleri* RNA-seq results

Sequencing summary	
Number of reads	
-non-mineralized teeth region	43,802,270
-mineralized teeth region	45,141,430
Assembly summary	
Number of transcripts	187,980
Average length of transcripts (bp)	705
N50 length (bp)	1,110
Maximum length of transcript (bp)	16,738
Mapping summary	
Total mapped reads	
-non-mineralized teeth	35,343,878
-mineralized teeth	35,511,782
% of Total mapped reads	
-non-mineralized teeth (%)	80.69
-mineralized teeth (%)	78.67

Table 2. List of mineralized cusp-specific proteins in *C. stelleri*

Transcript ID	Annotation ^a	Best hit organisms	E-value	FPKM (mineralized)	FPKM (non mineralized)	1st analysis		2nd analysis	
						Mascot scores	peptides	Mascot scores	peptides
Cs77024 c0_g1_i1	Neuroglobin-like	<i>Actinia tenebrosa</i>	2.00E-25	2323.65	2020.22	845	4	428	4
Cs77196 c0_g2_i1	Myoglobin	<i>Liolophura japonica</i>	3.00E-84	874.48	5345.03	387	4	234	3
Cs75674 c0_g4_i1	protein usf-like	<i>Octopus bimaculoides</i>	1.00E-101	67.74	2144.29	331	5	54	1
Cs82664 c0_g1_i1	Arginine kinase	<i>Liolophura japonica</i>	0	104.69	2469.32	189	4	178	2
Cs18149 c2_g1_i5	Peroxidasin	<i>Crassostrea gigas</i>	0	12.02	0.18	173	3	195	3
Cs68435 c0_g1_i1	No hits found			309.95	75	126	2	88	1
Cs83384 c0_g1_i1	protein yellow-like	<i>Lingula anatina</i>	3.00E-103	18.69	2.05	109	1	90	1
Cs18149 c2_g1_i2	Peroxidasin	<i>Mizuhopecten yessoensis</i>	1.00E-19	1.98	0	102	2	111	3
Cs62719 c1_g8_i1	gastric intrinsic factor-like protein 2	<i>Hyriopsis cumingii</i>	2.00E-35	28.05	0.08	114	1	56	1
Cs58153 c0_g2_i5	apoptosis inducing factor-3	<i>Haliotis discus discus</i>	0.00E+00	62.87	16.52	95	1	98	2
Cs22616 c0_g1_i1	copper transport protein ATOX1-like	<i>Exaiptasia pallida</i>	3.00E-27	2324.41	144.53	109	1	104	1
Cs28725 c1_g1_i1	hillarin	<i>Crassostrea virginica</i>	0	15.52	62.53	83	1	61	1
Cs84529 c0_g2_i1	muscle-specific protein 20- like isoform X2	<i>Lingula anatina</i>	7.00E-91	999.04	345.27	97	1	74	1
Cs59400 c0_g1_i1	Eukaryotic initiation factor 4A-II, partial	<i>Stegodyphus mimosarum</i>	0	145.13	141.93	95	1	85	1
Cs74134 c0_g2_i2	histone H2B 8-like	<i>Protobothrops mucrosquamatus</i>	2.00E-68	6.85	51.47	91	1	127	1
Cs46312 c2_g1_i1	hephaestin-like protein	<i>Stylophora pistillata</i>	0	0.27	179.74	90	1	84	2
Cs54305 c0_g1_i2	filamin-A-like isoform X1	<i>Biomphalaria glabrata</i>	0	125.51	91.37	75	1	147	2
Cs59318 c0_g1_i1	peroxiredoxin 6	<i>Cristaria plicata</i>	4.00E-119	274.95	561.11	77	2	93	2
Cs71263 c1_g1_i1	Peroxidasin	<i>Crassostrea gigas</i>	0	9.37	0.08	55	1	70	1
Cs69741 c0_g1_i1	superoxide dismutase (Cu- Zn)	<i>Drosophila sechellia</i>	6.00E-46	46.09	22.35	76	1	190	2
Cs53984 c1_g2_i8	filamin-A-like isoform X5	<i>Crassostrea virginica</i>	2.00E-104	10.13	4.09	69	1	132	2
Cs28687 c2_g1_i1	cytosolic malate dehydrogenase	<i>Mytilus galloprovincialis</i>	3.00E-176	34.88	81.1	61	1	59	1

^a Annotation is based on the results of BlastP analysis

Supplemental Data:

```

Cs90734lc0_g1_i1 -----
Liolophura japonica ferritin -----MKVLLVLAFLGLAFVAAEDKR-----
Cs22563lc0_g1_i1 -----ISPSFVKFITT VSSPINAGIHRKSAKMSV
Cs75144lc0_g1_i1 -----ISPSFVKFITT VSSPINAGIHRKSAKMSV
Cs17042lc0_g1_i1 RTSVFHKQNRFRAILTGTCFSCVSERTNKTVLNFNVLFFLISKVLFKLFENPKFVEMAG

Cs90734lc0_g1_i1 -----SYLTFLIQGHYYKTVDVLPGFSSFFKSIDDNLED
Liolophura japonica ferritin -LTCTAFSDSLIEKMNDQINLELHASYLYHGYARYFDRDDVALSGFADFFKHASSEEKDH
Cs22563lc0_g1_i1 SQPRQNYHAESEAGINKQINMELYASYVYQSMYYFDRDDVALKGFAKFFRESSKEEREH
Cs75144lc0_g1_i1 SQPRQNYHAESEAGINKQINMELYASYVYQSMYYFDRDDVALKGFAKFFRESSKEEREH
Cs17042lc0_g1_i1 TQPRQNFHKESEAGINKHINMELYASYAYQYMAYYFDRDDIALPGFVKLFKEHSDCCHTK
          :      :      *:. *:. * * * : * : *

Cs90734lc0_g1_i1 ADRFMEYLNKRGGRNIDYSVRIPDSCETVEQGLGELGAKRGACICQYISAGIVKKIGKT
Liolophura japonica ferritin ADKLMYYMNTRGCRFLLKD-ITYKDYCDKINEK--KPAELSSACICEFTAAA---TGGDP
Cs22563lc0_g1_i1 AEKFMKYQNKRGGRVVLQD-IKK-----
Cs75144lc0_g1_i1 AEKFMKYQNKRGGRVVLQD-IKK-----
Cs17042lc0_g1_i1 AEKLMKYQNKRGGRVILQD-VKK-----
          *:::* *:. * * : : :

Cs90734lc0_g1_i1 KGCKDSPMEWFNGLGGIEDALITERNMNHELLELLHQ--DAHDPHLEMFLETHYLGQSID
Liolophura japonica ferritin SSCSANRPEWFNGKQAMENALTIEHHYNDELLEKLR--STNDPHFEKFLLEDNYLQEQVN
Cs22563lc0_g1_i1 ---PERDEWDTGLKAMQAALELEKNVNOALLDLHKVADSHGDAQMMDFLEGEYLKEQVD
Cs75144lc0_g1_i1 ---PERDEWDTGLKAMQTALELEKNVNOALLDLHKVADSHGDAQMMDFLEGEYLKEQVD
Cs17042lc0_g1_i1 ---TDKNEFDTVLEAMHMSLTIEQTSNQALIDLHKIAEKTGDAAFADFLEGEFLKPAVD
          . * : . : : . : * * : * : * * : . * : * * : * : :

Cs90734lc0_g1_i1 VISKLARYITQIKKVGELGEYVFDKSL---
Liolophura japonica ferritin AIKELSDYITILKRTGDLGEYLFDKDLDSKY
Cs22563lc0_g1_i1 SIKQISDHITNLKRVGTGLGEYMFDKETLDD-
Cs75144lc0_g1_i1 SIKQISDHITNLKRVGTGLGEYMFDKETLDD-
Cs17042lc0_g1_i1 KIKKISDYMTNLKRVGPGLEGEYLFDKKTLLE-
          * : : : : * : * : * * * : * :

```

Figure S1 Multiple alignment of ferritins from *C. stelleri* and *L. japonica*. Amino acid sequences of *C. stelleri* ferritins were deduced from cDNA sequences. Conserved iron binding sites are boxed. Cs17042|c0_g1_i1 lacked the 5' terminus of the cDNA.

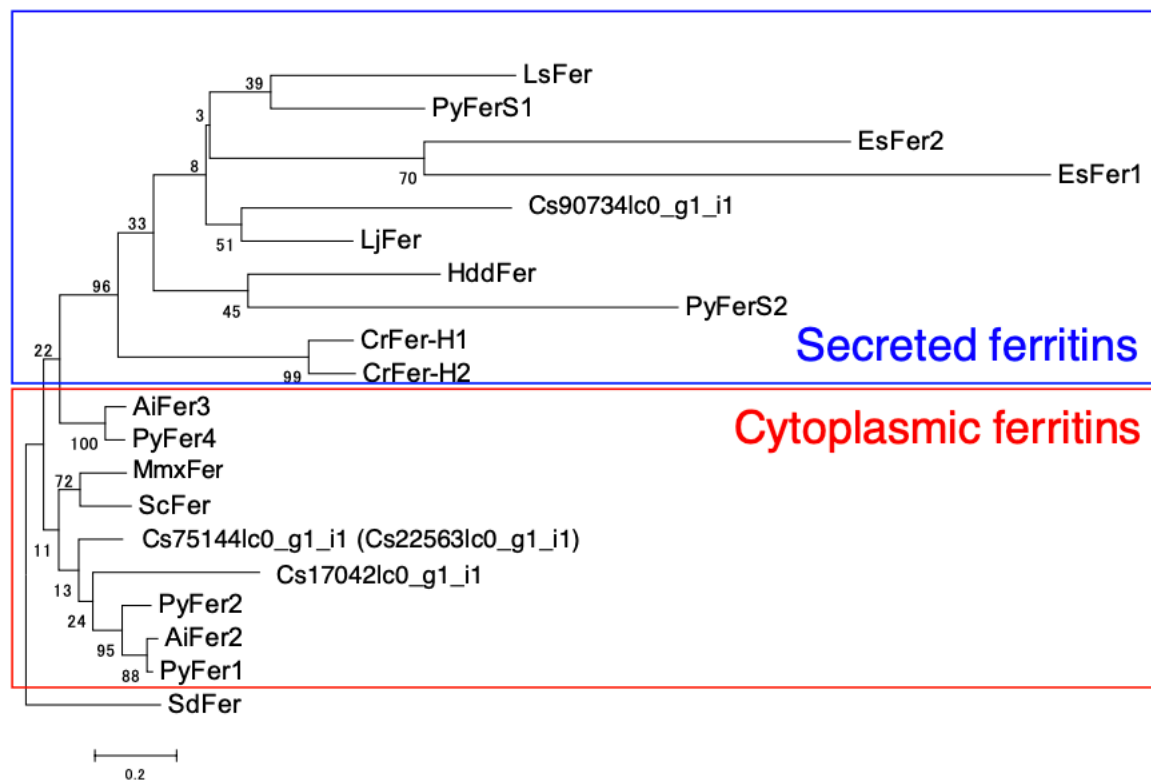


Figure S2 Phylogenetic tree of *C. stelleri* ferritins with other ferritins from Mollusca and Arthropoda.

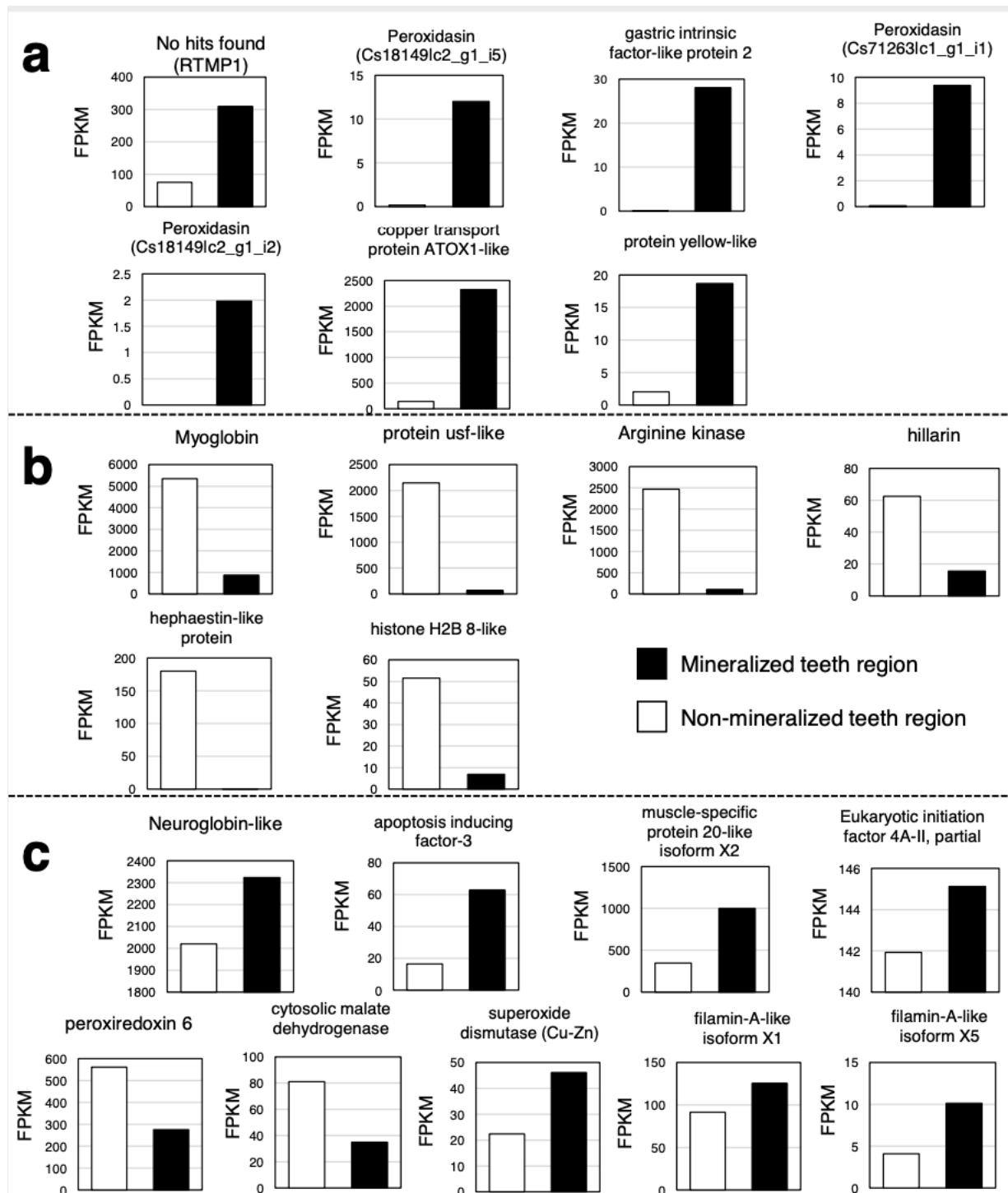


Figure S3 Expression of the transcripts encoding the mineralized cusp-specific proteins. (a) The transcripts that increased more than 4-fold at mineralized teeth region, (b) The transcripts that increased more than 4-fold at non-mineralized teeth region, (c) The transcripts differed in abundance by less than 4-fold between two regions.

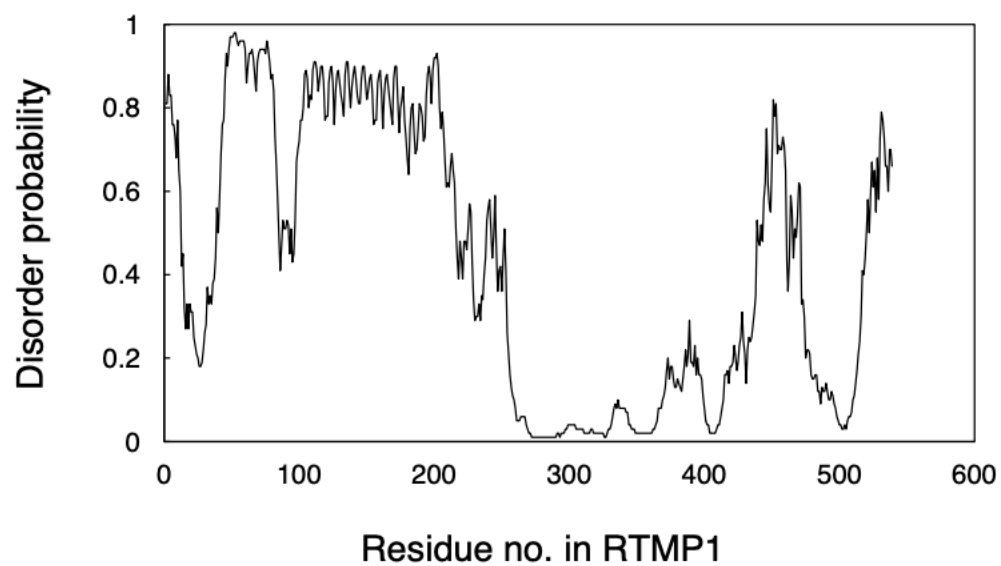


Figure S4 Disorder regions in RTMP1 were predicted with DISOPRED3⁷⁰.

Table S1. List of the twenty most highly expressed transcripts in non-mineralized teeth region

Transcript ID	Annotation ^a	Best hit organisms	E-value	Function
Cs79475 c0_g1_i1	peritrophin-1-like	<i>Lottia gigantea</i>	6.00E-76	Unknown
Cs17717 c0_g1_i5	No hits found			Unknown
Cs17717 c0_g1_i3	No hits found			Unknown
Cs17717 c0_g2_i1	No hits found			Unknown
Cs37223 c0_g1_i1	No hits found			Unknown
Cs70642 c0_g2_i1	PREDICTED: peritrophin-44-like	<i>Octopus bimaculoides</i>	4.00E-111	peritrophic membrane protein, chitin-binding
Cs75144 c0_g1_i1	ferritin subunit 2	<i>Hyriopsis schlegelii</i>	1.00E-102	Iron storage
Cs17717 c0_g1_i4	No hits found			Unknown
Cs17717 c0_g1_i1	No hits found			Unknown
Cs62747 c0_g1_i1	PREDICTED: cell migration-inducing and hyaluronan-binding protein isoform X2	<i>Erinaceus europaeus</i>	3.00E-180	Regulation of cell migration
Cs77196 c0_g2_i1	Myoglobin	<i>Liolophura japonica</i>	4.00E-79	Oxygen transport
Cs87163 c0_g1_i1	No hits found			Unknown
Cs47470 c2_g3_i1	Actin	<i>Aplysia californica</i>	0	Cytoskeletal protein
Cs37223 c0_g2_i1	No hits found			Unknown
Cs17717 c0_g1_i2	No hits found			Unknown
Cs76629 c0_g1_i1	cytochrome c oxidase subunit I (mitochondrion)	<i>Cryptochiton stelleri</i>	0	Energy metabolism.
Cs24354 c0_g1_i1	elongation factor 1 alpha	<i>Axinella verrucosa</i>	0	transcription
Cs56877 c0_g1_i1	Platelet glycoprotein Ib alpha chain, partial	<i>Stegodyphus mimosarum</i>	2.00E-14	surface membrane protein of platelets
Cs25220 c1_g1_i1	PREDICTED: protein PIF-like	<i>Octopus bimaculoides</i>	2.00E-85	Aragonite biomineralization, chitin-binding
Cs82664 c0_g1_i1	Arginine kinase	<i>Liolophura japonica</i>	0	phosphotransferase

^a Annotation is based on the results of BlastX analysis

Table S2. List of the twenty most highly expressed transcripts in mineralized teeth region

Transcript ID	Annotation ^a	Best hit organisms	E-value	Function
Cs58803 c0_g1_i1	hypothetical protein OXYTRI_13058 (macronuclear)	<i>Oxytricha trifallax</i>	1.00E-167	Unknown
Cs76629 c0_g1_i1	cytochrome c oxidase subunit I (mitochondrion)	<i>Cryptochiton stelleri</i>	0	Energy metabolism.
Cs43066 c0_g1_i1	cytochrome c oxidase subunit II (mitochondrion)	<i>Cryptochiton stelleri</i>	5.00E-149	Energy metabolism.
Cs26640 c0_g1_i1	cytochrome c oxidase subunit III (mitochondrion)	<i>Cryptochiton stelleri</i>	8.00E-148	Energy metabolism.
Cs43066 c1_g2_i1	ATP synthase F0 subunit 6 (mitochondrion)	<i>Cryptochiton stelleri</i>	2.00E-116	Energy metabolism.
Cs22243 c0_g6_i1	No hits found			Unknown
Cs22243 c0_g7_i3	No hits found			Unknown
Cs22243 c0_g7_i5	No hits found			Unknown
Cs22243 c0_g7_i6	No hits found			Unknown
Cs22243 c0_g7_i4	No hits found			Unknown
Cs45977 c0_g3_i1	hypothetical protein X975_22095, partial	<i>Stegodyphus mimosarum</i>	3.00E-19	Unknown
Cs45977 c0_g2_i1	hypothetical protein X975_22095, partial	<i>Stegodyphus mimosarum</i>	3.00E-18	Unknown
Cs22243 c0_g7_i2	No hits found			Unknown
Cs22243 c0_g5_i1	No hits found			Unknown
Cs71309 c7_g4_i1	antileukoproteinase-like	<i>Sorex araneus</i>	5.00E-09	Acid-stable proteinase inhibitor
Cs46263 c1_g1_i1	NADH dehydrogenase subunit 5 (mitochondrion)	<i>Cryptochiton stelleri</i>	0	Energy metabolism.
Cs50074 c0_g1_i1	NADH dehydrogenase subunit 1 (mitochondrion)	<i>Cryptochiton stelleri</i>	0	Energy metabolism.
Cs12250 c0_g1_i1	chitin binding beak protein 3	<i>Dosidicus gigas</i>	1.00E-09	defines the squid beak mechanical gradient
Cs46263 c0_g1_i1	cytochrome b (mitochondrion)	<i>Cryptochiton stelleri</i>	0	Energy metabolism.
Cs45977 c0_g1_i1	hypothetical protein OCBIM_22005804mg	<i>Octopus bimaculoides</i>	2.00E-25	Unknown

^a Annotation is based on the results of BlastX analysis

Table S3. Expression profiles of ferritin encoding transcripts in *C. stelleri*

Transcript ID	FPKM	
	Non-mineralized	Mineralized
Cs22563 c0_g1_i1	113.11	3.54
Cs75144 c0_g1_i1	8240.36	238.32
Cs17042 c0_g1_i1	15.29	0.71
Cs90734 c0_g1_i1	1.17	0.8

Table S4. List of the proteins identified from mineralized cusp fraction of radular teeth in *Cryptochiton stelleri*

Annotation*	Best hit organisms	E-value	1st analysis		2nd analysis		Transcript ID	FPKM (mineralized)	FPKM (non-mineralized)
			Mascot scores	Number of peptides	Mascot scores	Number of peptides			
Neuroglobin-like	<i>Actinia tenebrosa</i>	2.00E-25	845	4	428	4	Cs77024 c0_g1_i1	2323.65	2020.22
							Cs52177 c6_g1_i1	68.63	41.13
actin	<i>Modiolus modiolus</i>	1.00E-86	485	4			Cs75591 c0_g1_i1	2.78	0.29
Actin	<i>Aplysia californica</i>	0	529	6	237	4	Cs47470 c2_g3_i1	2810	5142.67
							Cs47470 c1_g1_i1	54.41	68.29
ependymin-related protein 1-like	<i>Lottia gigantea</i>	1.00E-33	525	6	345	5	Cs63606 c4_g2_i1	15.21	2.52
							Cs44691 c0_g1_i1	605.89	110.22
inter-alpha-trypsin inhibitor heavy chain H3-like isoform X2	<i>Lingula anatina</i>	3.00E-145	452	4	269	6	Cs47656 c0_g1_i2	17.7	2.27
							Cs47656 c0_g1_i1	112	2.24
filamin-A isoform X14	<i>Crassostrea gigas</i>	0	453	7	163	3	Cs22318 c0_g1_i1	66.57	125.82
tubulin beta chain	<i>Hydra vulgaris</i>	0	333	5	150	3	Cs54304 c1_g2_i5	101.68	647.37
							Cs71671 c4_g2_i1	1.33	0
							Cs71671 c4_g2_i3	0	1.4
							Cs71671 c4_g2_i4	0	1.49
							Cs71671 c4_g2_i5	0	7.36
Myoglobin	<i>Liolophura japonica</i>	3.00E-84	387	4	234	3	Cs77196 c0_g1_i1	75.14	140.63
							Cs78790 c0_g1_i1	29.05	124.08
							Cs77196 c0_g2_i1	874.48	5345.03
protein usf-like	<i>Octopus bimaculoides</i>	1.00E-101	331	5	54	1	Cs75674 c0_g4_i1	67.74	2144.29
							Cs81542 c0_g1_i1	0.78	45.96
alpha tubulin-like	<i>Lottia gigantea</i>	0			200	3	Cs88263 c0_g1_i1	189.83	1835.9
							Cs92361 c0_g1_i1	0	10.36
alpha tubulin-like	<i>Saccoglossus kowalevskii</i>	0	278	3	212	3	Cs88263 c0_g1_i2	603.6	1986.65
							Cs88263 c1_g1_i1	0.97	16.82
							Cs88263 c1_g1_i2	7.73	13.5
zonadhesin-like	<i>Parus major</i>	2.00E-26	147	2	139	3	Cs23761 c3_g1_i1	2473.16	14.36
							Cs19070 c4_g1_i2	39.29	1.14
fibrillin-2-like	<i>Lingula anatina</i>	0	250	4	150	3	Cs22298 c0_g1_i2	5.07	0.31
							Cs22298 c0_g1_i4	0.97	0.05
Arginine kinase	<i>Liolophura japonica</i>	0	189	4	178	2	Cs82664 c0_g1_i1	104.69	2469.32
							Cs79589 c0_g2_i1	0	16.41
							Cs79589 c0_g1_i1	1.66	22.46
CD109 antigen-like isoform X1	<i>Crassostrea gigas</i>	0	160	3			Cs41242 c0_g1_i2	333.61	2.37
							Cs41242 c0_g1_i1	22.1	0
Peroxidasin	<i>Crassostrea gigas</i>	0	173	3	195	3	Cs18149 c2_g1_i3	4.81	0
							Cs18149 c2_g1_i5	12.02	0.18
CD109 antigen-like isoform X2	<i>Crassostrea gigas</i>	0	163	3	398	9	Cs78436 c2_g1_i1	110.87	0.26
protein PIF-like	<i>Lottia gigantea</i>	1.00E-35			105	1	Cs53300 c0_g1_i1	0.3	0
							Cs53300 c0_g1_i2	1.94	0

tubulin beta-4B chain-like isoform X1	<i>Acanthaster planci</i>	0.00E+00	179	2	160	3	Cs71671 c1_g3_i1	2.59	4.37
							Cs71671 c1_g2_i1	54.54	211.44
inter-alpha-trypsin inhibitor heavy chain H4, partial	<i>Acanthisitta chloris</i>	1.00E-09	127	2	98	2	Cs48764 c0_g1_i1	1.01	0.26
uncharacterized protein LOC111636702	<i>Centruroides sculpturatus</i>	4.00E-05	138	2	86	1	Cs17650 c1_g2_i1	8.73	0
							Cs34146 c1_g1_i1	495.85	0.02
							Cs34146 c1_g1_i2	436.38	0.02
No hits found			93	1	233	4	Cs75160 c0_g1_i1	11.77	0
							Cs80392 c0_g1_i1	786.93	0
protein PIF isoform X2	<i>Crassostrea gigas</i>	1.00E-46			70	1	Cs55300 c1_g1_i1	1.38	0
No hits found			126	2	88	1	Cs68435 c0_g1_i2	56.26	316.81
							Cs68435 c0_g1_i1	309.95	75
							Cs11532 c0_g1_i1	1.11	1.95
							Cs93074 c3_g1_i1	5.43	4.9
lysosomal aspartic protease isoform X1	<i>Crassostrea gigas</i>	0	122	2			Cs54082 c4_g3_i5	70.7	40.45
							Cs64530 c0_g1_i1	1.22	0
							Cs64530 c0_g1_i3	0.81	0.59
protein yellow-like	<i>Lingula anatina</i>	3.00E-103	109	1	90	1	Cs83384 c0_g1_i1	18.69	2.05
melanotransferrin-like	<i>Aplysia californica</i>	8.00E-28	118	1			Cs33876 c0_g1_i1	55.79	46.56
							Cs33876 c0_g1_i2	104.35	35.82
							Cs35685 c0_g1_i1	4.7	1.26
cell migration-inducing and hyaluronan-binding protein-like	<i>Branchiostoma belcheri</i>	3.00E-125	116	1			Cs72782 c0_g1_i1	0.08	402.97
							Cs68459 c0_g1_i2	0.02	2.33
							Cs68459 c0_g1_i1	0	3.7
Peroxidasin	<i>Mizuhopecten yessoensis</i>	1.00E-19	102	2	111	3	Cs18149 c2_g1_i2	1.98	0
hypothetical protein LOTGIDRAFT_236297	<i>Lottia gigantea</i>	3.00E-40	72	1	67	1	Cs88032 c0_g1_i1	38.08	0.29
							Cs88032 c0_g1_i2	23.79	0.1
gastric intrinsic factor-like protein 2	<i>Hyriopsis cumingii</i>	2.00E-35	114	1	56	1	Cs62719 c1_g8_i1	28.05	0.08
apoptosis inducing factor-3	<i>Haliotis discus discus</i>	0.00E+00	95	1	98	2	Cs58153 c0_g2_i1	43.99	19.63
							Cs24699 c0_g2_i2	2.4	0
							Cs24699 c0_g2_i1	4.73	1.61
							Cs58153 c0_g2_i2	61.69	0
							Cs58153 c0_g2_i3	1.11	0
							Cs58153 c0_g2_i4	0	18.88
							Cs58153 c0_g2_i5	62.87	16.52
							Cs58153 c0_g2_i6	0	11.62
							Cs58153 c0_g2_i7	0	0
							Cs58153 c0_g2_i8	0	0
copper transport protein ATOX1-like	<i>Exaiptasia pallida</i>	3.00E-27	109	1	104	1	Cs22616 c0_g1_i1	2324.41	144.53
							Cs78473 c8_g1_i1	61.37	2.87
hillarin	<i>Crassostrea virginica</i>	0	83	1	61	1	Cs28725 c1_g1_i1	15.52	62.53
							Cs28725 c1_g1_i2	17.63	33.26
							Cs30794 c0_g1_i1	0	1.95

14-3-3 protein epsilon	<i>Dendroctonus ponderosae</i>	2.00E-148			101	1	Cs49664 c0_g1_i1	2.48	2.2
							Cs49664 c0_g2_i1	0	0
							Cs54082 c5_g1_i1	93.23	152.62
chitin binding beak protein 3	<i>Dosidicus gigas</i>	5.00E-10			52	1	Cs12250 c0_g1_i1	3170.58	0.28
							Cs71549 c0_g1_i1	87.72	0
muscle-specific protein 20-like isoform X2	<i>Lingula anatina</i>	7.00E-91	97	1	74	1	Cs50425 c0_g1_i2	17.41	6.06
							Cs84529 c0_g2_i1	999.04	345.27
Eukaryotic initiation factor 4A-II, partial	<i>Stegodyphus mimosarum</i>	0	95	1	85	1	Cs18720 c0_g1_i1	3.86	3.55
							Cs59400 c0_g1_i1	145.13	141.93
histone H2B 8-like	<i>Protobothrops mucrosquamatus</i>	2.00E-68	91	1	127	1	Cs55212 c0_g1_i2	4.08	0
							Cs55212 c0_g1_i1	1.61	0.49
							Cs74134 c0_g2_i1	0.52	15.62
							Cs74134 c0_g2_i2	6.85	51.47
							Cs74134 c0_g2_i4	0.31	9.28
							Cs74134 c0_g2_i5	3.21	2.81
							Cs74134 c0_g2_i6	7.35	3.45
							Cs74134 c0_g3_i1	2.27	2.79
lysosomal alpha-mannosidase	<i>Crassostrea gigas</i>	0			82	1	Cs17642 c2_g1_i1	42.49	36.06
							Cs26650 c0_g1_i2	0.28	0.4
neurexin-4-like	<i>Mizuhopecten yessoensis</i>	0			81	1	Cs29485 c0_g1_i1	37.55	40.55
							Cs43558 c0_g1_i1	1.23	0.42
ubiquitin family protein	<i>Okopleura dioica</i>	2.00E-128			79	1	Cs59998 c2_g1_i3	9.08	11.73
							Cs16655 c0_g1_i1	30.64	19.34
							Cs39013 c0_g1_i1	0	4.67
							Cs39013 c0_g2_i1	0.85	0.92
							Cs39013 c0_g3_i1	2.96	4.43
							Cs84216 c0_g1_i3	19.88	21.84
							Cs84800 c0_g1_i1	38.16	25
							Cs91127 c0_g1_i1	397.69	212.55
							Cs91127 c0_g2_i1	0	252.71
							Cs91127 c0_g3_i1	561.52	318.04
							Cs59998 c1_g1_i2	288.81	330.36
							Cs59998 c1_g1_i4	1234.58	652.14
							Cs84216 c0_g1_i2	2.17	1.83
							Cs59998 c1_g1_i1	2018.71	2111.01
							Cs84216 c0_g1_i1	53.85	152.12
							Cs59998 c1_g1_i3	248.35	304.91
							Cs59998 c2_g1_i2	13.27	8.81
							Cs59998 c2_g1_i5	2.34	2.23
							Cs59998 c2_g1_i1	23.16	9.17
							Cs59998 c2_g1_i4	0.55	0
							Cs13896 c0_g1_i1	705.54	508.63

hypothetical protein LOTGIDRAFT_171050	<i>Lottia gigantea</i>	2.00E-20	90	1			Cs27910 c6_g1_i2	3.71	1.05
							Cs85075 c0_g1_i1	289.36	45.69
							Cs85075 c0_g1_i2	304.44	118.11
							Cs85075 c0_g1_i3	122.72	14.49
							Cs27910 c6_g1_i1	10.98	1.82
							Cs85075 c0_g1_i4	105.51	15.21
hephaestin-like protein	<i>Stylophora pistillata</i>	0	90	1	84	2	Cs46312 c2_g1_i1	0.27	179.74
							Cs77488 c0_g2_i1	0	3.47
EGF-like domain-containing protein 2 isoform X3	<i>Octopus bimaculoides</i>	1.00E-25	89	1			Cs28544 c0_g2_i1	17.45	1.57
							Cs79867 c0_g1_i1	1.4	0.41
elongation factor 1 alpha	<i>Axinella verrucosa</i>	0	73	2	55	1	Cs24354 c0_g1_i1	1819.62	3625.82
							Cs83769 c0_g1_i1	29.02	47.26
cathepsin Z-like	<i>Mizuhopecten yessoensis</i>	2.00E-151	85	1			Cs50066 c0_g1_i1	130.55	55.51
							Cs38675 c0_g1_i1	2.76	1.09
gelsolin-like protein 2	<i>Acanthaster planci</i>	1.00E-155	84	1			Cs37973 c0_g2_i1	1.21	12.73
							Cs45844 c0_g1_i1	107.29	356.86
							Cs45844 c0_g1_i2	0	114.49
							Cs75047 c0_g2_i1	17.44	10.56
							Cs78032 c0_g1_i1	2.1	1.45
							Cs75047 c0_g1_i1	35	11.06
							Cs75047 c0_g1_i2	19.62	0
							Cs75047 c0_g1_i3	9.22	25.47
filamin-A-like isoform X1	<i>Biophalaria glabrata</i>	0	75	1	147	2	Cs54305 c0_g1_i2	125.51	91.37
							Cs54305 c0_g1_i1	5.54	9.2
							Cs86837 c0_g1_i1	2.92	1.5
radixin isoform X3	<i>Crassostrea gigas</i>	0	63	1			Cs19220 c1_g1_i1	162.74	221.31
							Cs68499 c0_g2_i1	3.74	4.87
filamin-A-like isoform X3	<i>Crassostrea virginica</i>	0.00E+00	78	1			Cs53984 c1_g2_i4	0	0
							Cs26775 c0_g1_i1	0.4	1.24
							Cs53984 c1_g2_i10	0	8.84
							Cs53984 c1_g2_i11	7.35	0
							Cs53984 c1_g2_i3	4.73	3.48
							Cs53984 c1_g2_i7	3.27	3.2
							Cs53984 c1_g2_i9	0.82	12.38
peroxiredoxin 6	<i>Cristaria plicata</i>	4.00E-119	77	2	93	2	Cs59318 c0_g1_i1	274.95	561.11
							Cs83444 c0_g1_i1	3.9	5.94
Peroxidasin	<i>Crassostrea gigas</i>	0	55	1	70	1	Cs71263 c1_g1_i1	9.37	0.08
							Cs71263 c1_g1_i8	6.11	0.07
galaxin-like	<i>Acropora digitifera</i>	1.00E-12	71	1			Cs12196 c0_g1_i1	107.08	0.07
							Cs13013 c0_g1_i1	4.34	0
							Cs76396 c0_g2_i1	9.68	43.42
							Cs73246 c0_g2_i1	0	0.81

thioredoxin peroxidase	<i>Artemia franciscana</i>	1.00E-116	70	1			Cs73246 c0_g3_i1	0.33	0.38
							Cs73246 c0_g4_i1	0.47	0
							Cs76396 c0_g1_i1	3.63	41.8
							Cs73246 c0_g1_i1	0.5	1.06
							Cs76396 c0_g3_i1	10.78	3.51
							Cs76396 c0_g4_i1	17	31.17
							Cs76396 c0_g5_i1	7.64	16.82
superoxide dismutase (Cu-Zn)	<i>Drosophila sechellia</i>	6.00E-46	76	1	190	2	Cs69741 c0_g1_i1	46.09	22.35
retrograde protein of 51 kDa-like isoform X4	<i>Crassostrea virginica</i>	3.00E-136	70	1	70	1	Cs20050 c0_g2_i1	33.69	1.12
							Cs34327 c0_g1_i1	1683.79	60.17
peptidyl-prolyl cis-trans isomerase-like	<i>Stylophora pistillata</i>	1.00E-89	57	1			Cs67145 c0_g1_i1	319.14	362.41
							Cs30224 c0_g1_i1	6.07	4.7
filamin-A-like isoform X5	<i>Crassostrea virginica</i>	2.00E-104	69	1	132	2	Cs53984 c1_g2_i1	4.69	3.34
							Cs53984 c1_g2_i6	6.78	5.64
							Cs53984 c1_g2_i8	10.13	4.09
CKLF-like MARVEL transmembrane domain-containing protein 4	<i>Lingula anatina</i>	8.00E-38			97	1	Cs93043 c0_g1_i1	9.57	0
							Cs93043 c0_g1_i2	6.74	5.05
Profilin	<i>Tuber melanosporum Mel28</i>	3.00E-10			67	1	Cs35744 c0_g2_i1	4.1	4.57
							Cs35744 c0_g1_i1	1.07	0
							Cs93141 c0_g2_i1	168.38	243.11
triosephosphate isomerase-like	<i>Branchiostoma belcheri</i>	1.00E-133			66	1	Cs62667 c1_g1_i1	24.59	63.64
							Cs41497 c0_g2_i1	0.16	0.72
							Cs41497 c0_g3_i1	0.16	0
							Cs41497 c0_g1_i1	0.16	1.08
CD109 antigen-like	<i>Lottia gigantea</i>	0			64	1	Cs93025 c0_g1_i1	7.22	21.47
clathrin heavy chain 1 isoform X2	<i>Mizuhopecten yessoensis</i>	0	64	1			Cs59011 c0_g1_i2	21.69	33.63
							Cs59011 c0_g1_i1	16.73	24.6
ubiquitin, partial (macronuclear)	<i>Nyctotherus ovalis</i>	7.00E-72	61	1			Cs70580 c0_g2_i1	1.53	0
cytosolic malate dehydrogenase	<i>Mytilus galloprovincialis</i>	3.00E-176	61	1	59	1	Cs28687 c2_g1_i1	34.88	81.1
							Cs21624 c0_g1_i1	0.94	1.56
ADP-ribosylation factor 1-like isoform X3	<i>Saccoglossus kowalevskii</i>	2.00E-117			58	1	Cs22952 c1_g1_i1	2.86	3.71
							Cs87098 c0_g1_i1	125.7	195.08
14-3-3 protein zeta isoform X1	<i>Dinoponera quadricaps</i>	1.00E-130			53	1	Cs74650 c0_g1_i1	182.51	195.15
							Cs84401 c0_g1_i1	5.71	4.62
nucleolin-like	<i>Lingula anatina</i>	8.00E-31			52	1	Cs73306 c0_g1_i3	113.67	238.35
							Cs21433 c0_g1_i2	3.39	4.94
							Cs21433 c0_g1_i4	0.93	0.37
							Cs73306 c0_g1_i2	3.5	4.1
							Cs21433 c0_g1_i1	0	0.34
							Cs73306 c0_g1_i1	27.18	29.24
F-actin-capping protein subunit alpha-2-like	<i>Lingula anatina</i>	6.00E-169			52	1	Cs83170 c0_g1_i1	37.69	72.2
							Cs31098 c0_g1_i1	1.04	1.99

tyrosine-protein kinase receptor Tie-1-like	<i>Lingula anatina</i>	4.00E-09	57	1		Cs33897 c6_g1_i7	26.47	44.23
						Cs33897 c4_g1_i3	0.56	0
						Cs33897 c4_g1_i4	1.7	0
						Cs33897 c4_g1_i5	0	0.44
						Cs33897 c6_g1_i4	68.56	21.8
						Cs33897 c4_g1_i2	0.74	1.78
						Cs33897 c6_g1_i1	50.57	79
						Cs33897 c6_g1_i6	50.98	49.12
						Cs33897 c6_g1_i5	47.34	0
						Cs33897 c6_g1_i2	33.26	0
NADP-dependent malic enzyme	<i>Crassostrea gigas</i>	0	57	1		Cs92452 c0_g1_i1	2.44	8.79
dipeptidyl peptidase 1-like isoform X1	<i>Crassostrea virginica</i>	0.00E+00	55	1		Cs92769 c1_g1_i1	70.84	14.85
						Cs28054 c0_g1_i1	2.33	0.3
nucleoside diphosphate kinase A	<i>Crassostrea gigas</i>	1.00E-76	54	1		Cs27778 c0_g1_i1	427.75	93.45
						Cs27778 c0_g2_i1	0	650.63
						Cs67705 c0_g1_i1	0	11.49
						Cs67705 c0_g2_i1	9.01	0.85
Phosphoenolpyruvate carboxykinase [GTP]	<i>Lottia gigantea</i>	0.00E+00	53	1		Cs63416 c0_g1_i1	0.72	1.31
						Cs81194 c0_g1_i1	22.04	31.08
alpha-L-fucosidase-like	<i>Mizuhopecten yessoensis</i>	0	51	1		Cs82886 c4_g3_i2	2.95	30.29
						Cs82886 c4_g3_i4	4.73	0
collagen alpha-1(XII) chain-like	<i>Octopus bimaculoides</i>	5.00E-24	51	1		Cs24988 c0_g1_i3	1.83	0.12
						Cs24988 c0_g1_i4	0.42	0
						Cs24988 c0_g1_i7	0	0
						Cs24988 c0_g1_i8	10.12	1.23

* Annotation is based on the results of BlastP analysis

Table S5. List of the proteins identified from base plus membrane fraction of radular teeth in *Cryptochiton stelleri*

Annotation ^a	Best hit	E-value	1st analysis		2nd analysis		Transcript ID	FPKM (mineralized)	FPKM (non-mineralized)
			Mascot scores	number of peptides	Mascot scores	number of peptides			
inter-alpha-trypsin inhibitor heavy chain H3-like isoform X2	<i>Lingula anatina</i>	3.00E-145	239	3	81	2	Cs47656 c0_g1_i2	17.7	2.27
							Cs47656 c0_g1_i1	112	2.24
CD109 antigen-like isoform X2	<i>Crassostrea gigas</i>	0	233	5	490	10	Cs78436 c2_g1_i1	110.87	0.26
zonadhesin-like	<i>Parus major</i>	2.00E-26	233	3	165	2	Cs19070 c4_g1_i2	39.29	1.14
							Cs23761 c3_g1_i1	2473.16	14.36
alpha tubulin-like	<i>Lottia gigantea</i>	0			134	2	Cs81533 c0_g1_i1	1.48	1.05
							Cs81533 c0_g2_i1	0	1.22
							Cs88263 c0_g1_i1	189.83	1835.9
							Cs88263 c0_g1_i3	901.4	732.35
							Cs92361 c0_g1_i1	0	10.36
							Cs92361 c1_g3_i1	18.12	0.22
hypothetical protein	<i>Lottia gigantea</i>	2.00E-50	220	4	79	2	Cs40476 c0_g1_i1	6.45	92.83
							Cs83855 c0_g1_i1	0.13	2.38
Actin	<i>Aplysia californica</i>	0	216	3	99	3	Cs47470 c1_g1_i1	54.41	68.29
							Cs47470 c2_g3_i1	2810	5142.67
actin	<i>Modiolus modiolus</i>	1.00E-86	194	1			Cs75591 c0_g1_i1	2.78	0.29
CD109 antigen-like isoform X1	<i>Crassostrea gigas</i>	0	191	2	61	1	Cs41242 c0_g1_i1	22.1	0
							Cs41242 c0_g1_i2	333.61	2.37
							Cs70978 c0_g1_i1	21.54	0
							Cs84232 c0_g1_i1	11.94	0
retrograde protein of 51 kDa-like isoform X4	<i>Crassostrea virginica</i>	3.00E-136	137	2	178	3	Cs20050 c0_g2_i1	33.69	1.12
							Cs34327 c0_g1_i1	1683.79	60.17
No hits found			129	1	68	1	Cs32565 c1_g1_i1	40.95	0
							Cs32565 c1_g1_i2	5.45	0.67
malate dehydrogenase, mitochondrial isoform X2	<i>Aplysia californica</i>	0	121	1			Cs75179 c0_g1_i1	77.41	43.32
							Cs75179 c0_g1_i2	0	10.36
fibrillin-2-like	<i>Lingula anatina</i>	0	113	3	62	1	Cs22298 c0_g1_i2	5.07	0.31
							Cs22298 c0_g1_i4	0.97	0.05
uncharacterized protein LOC111636702	<i>Centruroides sculpuratus</i>	4.00E-05	105	2	263	3	Cs17650 c1_g2_i1	8.73	0
							Cs34146 c1_g1_i1	495.85	0.02
							Cs34146 c1_g1_i2	436.38	0.02
cell surface protein	<i>Corynebacterium phoceense</i>	2.00E-06	94	1			Cs58933 c0_g1_i1	2.91	0
							Cs65631 c0_g1_i1	27.59	1.17
peroxidase-like	<i>Lottia gigantea</i>	1.00E-152	80	1			Cs81561 c0_g1_i1	1.51	0.35
							Cs11665 c0_g1_i1	6.87	74.38
							Cs49435 c0_g1_i1	3.19	193.6
							Cs54304 c1_g2_i1	110.53	2
							Cs54304 c1_g2_i2	53.84	69.25
							Cs54304 c1_g2_i3	6.3	104.23

tubulin beta chain	<i>Hydra vulgaris</i>	0	70	1	62	1	Cs54304 c1_g2_i4	0	0
							Cs54304 c1_g2_i5	101.68	647.37
							Cs54304 c1_g2_i6	2.43	19.67
							Cs54304 c1_g4_i1	0.16	36.87
							Cs71671 c1_g2_i1	54.54	211.44
							Cs71671 c1_g3_i1	2.59	4.37
							Cs71671 c1_g3_i2	76.04	1.78
							Cs71671 c1_g3_i3	0	0
							Cs71671 c1_g3_i4	14.97	10.72
							Cs71671 c4_g2_i1	1.33	0
							Cs71671 c4_g2_i3	0	1.4
							Cs71671 c4_g2_i8	0.74	0
							Cs71671 c4_g3_i1	1.05	3.49
							Cs71671 c4_g2_i2	3.3	4.2
							Cs71671 c4_g2_i4	0	1.49
							Cs71671 c4_g2_i5	0	7.36
							Cs71671 c4_g2_i9	0.68	0
No hits found			62	1			Cs43469 c0_g1_i1	2.01	0
							Cs85396 c0_g1_i1	151.1	0
collagen alpha-1(XII) chain-like	<i>Octopus bimaculoides</i>	9.00E-28	61	1			Cs24988 c0_g1_i1	2.58	0
							Cs24988 c0_g1_i11	0.62	0.15
							Cs24988 c0_g1_i5	12.64	6.02
							Cs24988 c0_g1_i6	0	0
uncharacterized protein LOC110454775	<i>Mizuhopecten yessoensis</i>	3.00E-24	57	1			Cs61402 c0_g1_i1	2	2.88
							Cs61402 c0_g1_i2	7.16	1.49
							Cs75949 c0_g1_i1	379.51	272.39
alpha tubulin-like	<i>Saccoglossus kowalevskii</i>	0	56	1	126	2	Cs20163 c0_g1_i1	111.79	165.29
							Cs36028 c0_g1_i1	0	17.03
							Cs36028 c0_g2_i1	0	0
							Cs36028 c0_g3_i1	0	0
							Cs43007 c0_g2_i1	0.44	30.38
							Cs43007 c0_g4_i1	16.57	2.92
							Cs64094 c0_g1_i1	0	10.52
							Cs64094 c0_g1_i2	4.73	34.2
							Cs72036 c0_g1_i1	1.42	1.72
							Cs88263 c0_g1_i1	189.83	1835.9
							Cs88263 c0_g1_i2	603.6	1986.65
							Cs88263 c1_g1_i1	0.97	16.82
							Cs88263 c1_g1_i2	7.73	13.5
							Cs88263 c1_g1_i3	6.88	11.91
							Cs88573 c0_g1_i1	0.66	3.55
							Cs92361 c0_g1_i1	0	10.36

							Cs92361 c0_g1_i2	0.76	1.88
No hits found			54	1			Cs78604 c1_g2_i2	20	0.68
chitin binding beak protein 3	<i>Dosidicus gigas</i>	5.00E-10	53	1	65	1	Cs12250 c0_g1_i1	3170.58	0.28
							Cs71549 c0_g1_i1	87.72	0
No hits found			52	1	51	1	Cs17717 c0_g1_i3	38.82	11750.12
							Cs17717 c0_g1_i4	105.68	7613.02
							Cs17717 c1_g1_i1	0.63	122.76
							Cs17717 c1_g1_i4	0.58	206.04
							Cs17717 c0_g1_i2	21	3943.71
							Cs17717 c0_g1_i1	41.73	6108.95
No hits found					122	2	Cs75160 c0_g1_i1	11.77	0
							Cs80392 c0_g1_i1	786.93	0
putative cysteine proteinase CG12163	<i>Branchiostoma belcheri</i>	9.00E-33			110	1	Cs85860 c0_g2_i1	4.05	0
uncharacterized protein LOC106869807	<i>Octopus bimaculoides</i>	6.00E-09			87	1	Cs35465 c0_g1_i1	73.35	3.38
							Cs62172 c0_g1_i1	2.54	0
tropomyosin-1 isoform X19	<i>Crassostrea virginica</i>	8.00E-65			68	1	Cs85863 c1_g1_i3	0	26.24
							Cs21476 c0_g1_i1	2.56	2.92
							Cs21476 c0_g1_i2	2.5	0.29
							Cs85863 c1_g1_i1	150.91	107.33
							Cs85863 c1_g1_i2	35.78	4.81
ependymin-related protein 1-like	<i>Lottia gigantea</i>	1.00E-33			64	1	Cs63606 c4_g1_i1	0	0
							Cs63606 c4_g2_i1	15.21	2.52
							Cs44691 c0_g1_i1	605.89	110.22
filamin-A isoform X14	<i>Crassostrea gigas</i>	0			62	1	Cs13902 c0_g1_i1	0.57	2.96
							Cs22318 c0_g1_i1	66.57	125.82
transcription factor with AP2 domain(s), putative	<i>Plasmodium malariae</i>	2.00E-04			60	1	Cs22247 c2_g1_i1	331.87	0.21
							Cs22247 c2_g1_i3	123	0
							Cs22247 c3_g1_i12	0	0
							Cs22247 c3_g1_i3	4.24	0
							Cs22247 c3_g1_i9	2.98	0
inter-alpha-trypsin inhibitor heavy chain H4, partial	<i>Acanthisitta chloris</i>	1.00E-09			56	1	Cs48764 c0_g1_i1	1.01	0.26
hypothetical protein LOTGIDRAFT_236297	<i>Lottia gigantea</i>	3.00E-40			54	1	Cs88032 c0_g1_i1	38.08	0.29
							Cs88032 c0_g1_i2	23.79	0.1

^a Annotation is based on the results of BlastP analysis

Table S6. Localization analysis of mineralized cusp-specific proteins

Transcript ID	Annotation ^a	Signal sequence prediction software				
		SignalP	TargetP ^b	PSORT	MultiLoc2	Signal-BLAST
Cs77024 c0_g1_i1	Neuroglobin-like			cytosol	cytoplasmic	
Cs77196 c0_g2_i1	Myoglobin			cytosol	secretory pathway	
Cs75674 c0_g4_i1	protein usf-like			mitochondria	secretory pathway	○
Cs82664 c0_g1_i1	Arginine kinase		M	mitochondria	cytoplasmic	
Cs18149 c2_g1_i5	Peroxidasin	○	S	plasma membrae	secretory pathway	○
Cs68435 c0_g1_i1	No hits found		S	extracellular	secretory pathway	○
Cs83384 c0_g1_i1	protein yellow-like	○	S	plasma membrae	secretory pathway	○
Cs18149 c2_g1_i2 ^c	Peroxidasin	○	S	plasma membrae	secretory pathway	○
Cs62719 c1_g8_i1	gastric intrinsic factor-like protein 2	○	S	extracellular	secretory pathway	○
Cs58153 c0_g2_i5	apoptosis inducing factor-3			mitochondria	secretory pathway	
Cs22616 c0_g1_i1	copper transport protein ATOX1-like			extracellular	cytoplasmic	
Cs28725 c1_g1_i1	hillarin		M	mitochondria	cytoplasmic	
Cs84529 c0_g2_i1	muscle-specific protein 20-like isoform X2			cytosol	cytoplasmic	
Cs59400 c0_g1_i1	Eukaryotic initiation factor 4A-II, partial			nucleus	nuclear	
Cs74134 c0_g2_i2 ^c	histone H2B 8-like			nucleus	nuclear	
Cs46312 c2_g1_i1	hephaestin-like protein		S	plasma membrae	secretory pathway	○
Cs54305 c0_g1_i2 ^c	filamin-A-like isoform X1			nucleus	cytoplasmic	
Cs59318 c0_g1_i1	peroxiredoxin 6			cytosol	cytoplasmic	
Cs71263 c1_g1_i1	Peroxidasin	○	S	extracellular	secretory pathway	
Cs69741 c0_g1_i1	superoxide dismutase (Cu-Zn)	○	S	extracellular	secretory pathway	○
Cs53984 c1_g2_i8	filamin-A-like isoform X5			cytosol	cytoplasmic	
Cs28687 c2_g1_i1	cytosolic malate dehydrogenase			mitochondria	cytoplasmic	

^a Annotation is based on the results of BlastP analysis^b S, Secretory pathway; M, Mitochondrion^c cDNA of those proteins are likely truncated.

Future Work

Overall, we were successful in this project. However, more work is needed, including identifying mechanisms of transformation from ferrihydrite to magnetite, identification of proteins in both fully mineralized teeth as well as proteins that might be involved in mineral transport from epithelial tissue and the stylus into the teeth. In addition, investigation of other species of chiton teeth with a different ecology will shed light on both design elements as well as chemistry involved in forming specific phases of mineral in these incredibly wear resistant teeth. Translation and validation using biomimetics still remains a high priority as well.

Supported Personnel and Collaborations:
Training, Education, and Outreach Achievements

Training, Education, and Outreach Achievements

UCR:

1. Mr. Steven Herrera (BS, UCR and underrepresented minority), was a Ph.D. student working on the project. He was mentored by Kisailus.

2. Dr. Anna Pohl was a postdoc partially supported on this project. She was mentored by Kisailus.

We have had more than 7 groups of high school and community college students tour our lab in the past year.

Dr. Michiko Nemoto is a former Kisailus post-doc and now a collaborator in this project. She is now a professor at Okayama University. She was mentored as a professor by Kisailus.

Dr. Omar Avila-Poveda is a collaborator at the Universidad of Sinaloa in Mexico.

Publications:

“Integrated transcriptomic and proteomic analyses of a molecular mechanism of radular teeth biomineralization in *Cryptochiton stelleri*,” Michiko Nemoto, Dongni Ren, Steven Herrera, Songqin Pan, Takashi Tamura, Kenji Inagaki, David Kisailus, *Scientific Reports*, 9 (2019), 856. DOI: 10.1038/s41598-018-37839-2

Two additional manuscripts are in preparation.

Presentations:

“Biological Crystallization of Ultrahard Teeth and Translation to Multi-Functional Materials,” **Invited talk.** 19th International Conference on Crystal Growth and Epitaxy (ICCGE-19). Keystone, Colorado, July 28–August 2, 2019.

“Biological Crystallization of Ultrahard Teeth and Translation to Multi-Functional Materials,” **Invited talk.** Collaborative Conference on Materials Research. Seoul, South Korea. June 25-29, 2019.

“Biologically Inspired Multi-Functional Materials,” **Invited seminar.** Lawrence Berkeley National Labs. Berkeley, CA. April 17, 2019.

“Biological Crystallization of Ultrahard Teeth and Translation to Multi-Functional Materials,” **Invited talk.** 43rd International Conference and Exposition on Advanced Ceramics Conference, Daytona Beach, FL, January, 2019.

“Biologically Inspired Multi-Functional Materials For Structural, Energy and Water-Related Applications”, **Invited seminar**. Chemistry Department, Sun Yat Sen University. Guangzhou, China. December, 2018.

“Biologically Inspired Multi-Functional Materials,” **Invited seminar**. Aerospace Engineering Department, University of Illinois at Urbana-Champaign. Urbana, IL. October 29, 2018.

“Materials Inspired from Fossils and Their Relatives,” **Invited talk**. Materials Science and Engineering Congress (MSE). Darmstadt, Germany. September 26-28, 2018.

“Fiber Functionality in Nature and Beyond: Assembly, Templating and Toughening,” **Keynote lecture**. Global Innovation Research Symposium. Tokyo, Japan. September 20, 2018.

“Biological Synthesis and Structural Developments in the Ultrahard Teeth of Chiton,” **Invited talk**. 6th International Meeting on Magnetotactic Bacteria. Kanazawa, Japan. September 10-14, 2018.

“Multifunctional Materials for Structural, Energy and Water Related Applications,” **Invited talk**. International Materials Research Congress. Cancun, Mexico. August 21, 2018.