AAOF Final Report

Type of Award: Postdoctoral Fellowship Award

Principal Investigator: Alice Goodwin

Title of Project: Genetics of temporomandibular joint development and growth

Period of AAOF Support: 07/01/16-06/30/18, No cost extension 07/01/18-3/31/19

Amount of Funding: $100,000

Summary/Abstract of Completed Project Results:
Costello syndrome (CS) is a rare congenital anomaly syndrome characterized by craniofacial malformations, dermatologic anomalies, cardiac defects, musculoskeletal abnormalities, growth delay, and cognitive deficits, and nearly all patients have a heterozygous, *de novo* germline mutation in *HRAS* that results in a constitutively active Ras signaling. We found that individuals with CS have a higher incidence of class III malocclusion (37% compared to 6% in the general population, p=.0001), and the class III malocclusion in our cohort was skeletal in nature and primarily due to maxillary hypoplasia. In order to further understand the malocclusion in CS and the contribution of Ras signaling to class III malocclusion, we utilized the CS mouse model, carrying an *HRas*$^{G12V}$ mutation, which phenocopies many of the characteristics of CS including the craniofacial and dental defects. We found there is a decrease in length of the cranial base and maxilla and early fusion of the intersphenoid synchondrosis (ISS) in the CS mouse compared to control which may contribute to the maxillary hypoplasia in CS. In addition, we found the major difference in the shape of the CS mandible was an increase in the width of the condyles which matched the increased width of the skull. Also, there was narrowing in the medio-lateral and antero-posterior widths and flattening in the dorso-ventral height of the condylar heads. There was also erosion and pitting on the CS condylar heads, and the relative mineral bone density of the CS mandible, condyle, and skull was decreased compared to control. Finally, the mandibular condylar cartilage was thinner with decreased proliferative and hypertrophic chondrocytes. These data suggest the development of the mandible is normal in CS; however, the mandible adapts and changes shape secondarily to widening of the skull and shortening of the maxilla and cranial base in CS. Overall, this project shed light on the role of Ras signaling in the development of class III malocclusion, specifically the growth of the mandible and maintenance of the MCC.

Response to the following questions:

1. Were the original, specific aims of the proposal realized?
   Yes, at the point when the progress report was submitted, the specific aims were modified slightly based on the reviewers' comments on the AAOF PFA application and progress on the proposed specific aims, and these revised specific aims were

pursued. We focused on malocclusion in CS individuals and mice in order to narrow the scope of the project.

2. Were the results published?
   The results have not been published; however, we are currently putting the data together in a manuscript.
   a. If so, cite reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers
      Not available.
   b. Was AAOF support acknowledged?
      AAOF support will certainly be acknowledged in all future publications from my postdoctoral work.
   c. If not, are there plans to publish? If not, why not?
      Yes, there are plans to publish the work.

3. Have the results of this proposal been presented?
   The results of the proposal have not been presented.
   a. If so, list titles, author or co-authors of these presentation/s, year and locations
   b. Was AAOF support acknowledged?
   c. If not, are there plans to do so? If not, why not?
      We plan to present the data at future meetings.

4. To what extent have you used, or how do you intend to use, AAOF funding to further your career?
   AAOF funding was instrumental in advancing my career. It provided resources for my postdoctoral training in the lab, during which I successfully obtained an NIDCR K08 award which began July 1, 2018. I also secured a tenure track, assistant professor position in the UCSF School of Dentistry, Department of Orofacial Sciences, Division of Craniofacial Anomalies which began January 1, 2018. The AAOF PFA support enabled me to both continue to develop my research project and practice clinical orthodontics which has allowed me to secure additional NIH funding and a faculty position, developing my research program in craniofacial anomalies and treating patients, and so I am very grateful for the support of the AAOF PFA.

Accounting for Project: All of the funds were utilized. There are no remaining funds.

## INTRODUCTION

Costello syndrome (CS) is a rare congenital anomaly syndrome characterized by craniofacial malformations, dermatologic anomalies, cardiac defects, musculoskeletal abnormalities, growth delay, and cognitive deficits, and nearly all patients have a heterozygous, *de novo* germline mutation in *HRAS* that results in constitutively active Ras signaling. In a previous study, we found that individuals with CS have a higher incidence of class III malocclusion (37% compared to 6% in the general population, p=.0001). The goal of the project during the AAOF PFA was to analyze the malocclusion in CS individuals and a CS mouse model in order to understand the role of Ras signaling in development of class III malocclusion, specifically whether activated Ras signaling causes mandibular prognathism and temporomandibular joint (TMJ) dysfunction in CS.

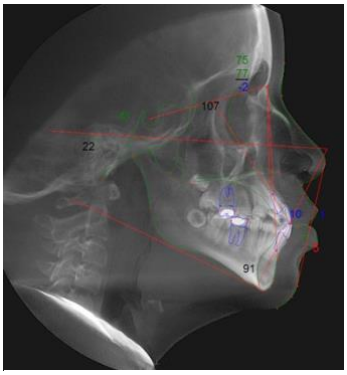


Figure 1. Lateral cephalogram and tracing of a 12 year old female with CS showing negative sagittal jaw relationship due to a hypoplastic maxilla (SNA=75°) and increased vertical jaw relationship typical of CS.

## STUDIES AND RESULTS

### Aim 1: Analyze the shape of the condyle in CS individuals compared to normal individuals.

CS is an extremely rare syndrome (~200 reported cases), and so we recruited most subjects at the CS family conferences that are held every 2 years, which bring CS individuals and their families together from around the country. I attended the CS/CFC family conference July 25-30, 2017 in Orlando, FL. We examined 30 CS individuals, performing clinical examinations and collecting intra- and extra-oral photographs to diagnose malocclusion. I had planned to also evaluate symptoms of temporomandibular joint disorders in this cohort, however, I found that most of the CS individuals who attended the

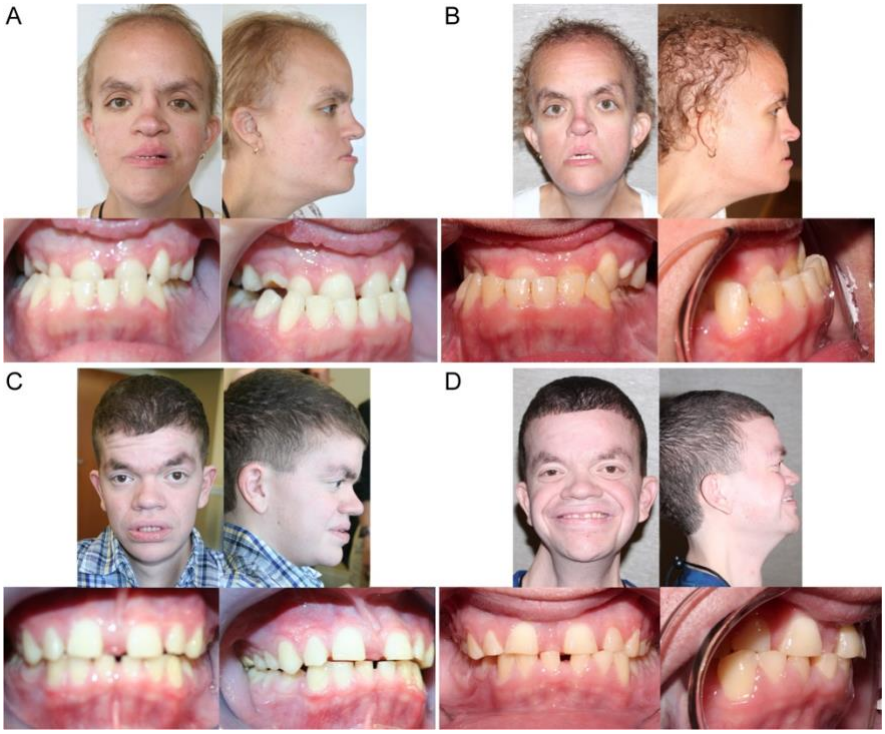


Figure 2. Images of a female with CS at 23 (A) and 31 (B) years of age showing the progression of class III malocclusion with increase in negative overjet and compensatory hyper-eruption of the mandibular incisors, and a male with CS at 20 (C) and 28 (D) years of age showing worsening of mandibular asymmetry, with the mandibular midline shifting to the left, and increasing incisal wear on the teeth.

conference were young (under the age of 5, 16/30=53%), and so it was very difficult to evaluate symptoms of joint dysfunction. Furthermore, we were unable to diagnose in many of the subjects due to cognitive delay, which made clear reporting of TMD symptoms impossible.

Despite contacting families prior to the conference to bring CBCT or CT imaging, it was challenging to obtain a significant number of X-rays in large part because CS individuals do not necessarily seek orthodontic treatment, most often because the individual cannot tolerate orthodontic appliances. From the data we were able to attain, **the class III malocclusion in CS individuals is skeletal, and primarily due to maxillary hypoplasia** (Figure 1).

Additionally, we collected records on several subjects in 2009, 2013, and 2017, and so we have records of the **natural progression of malocclusion in CS individuals, which show that the class III malocclusion and asymmetry worsen over time** (Figure 2; N=6).

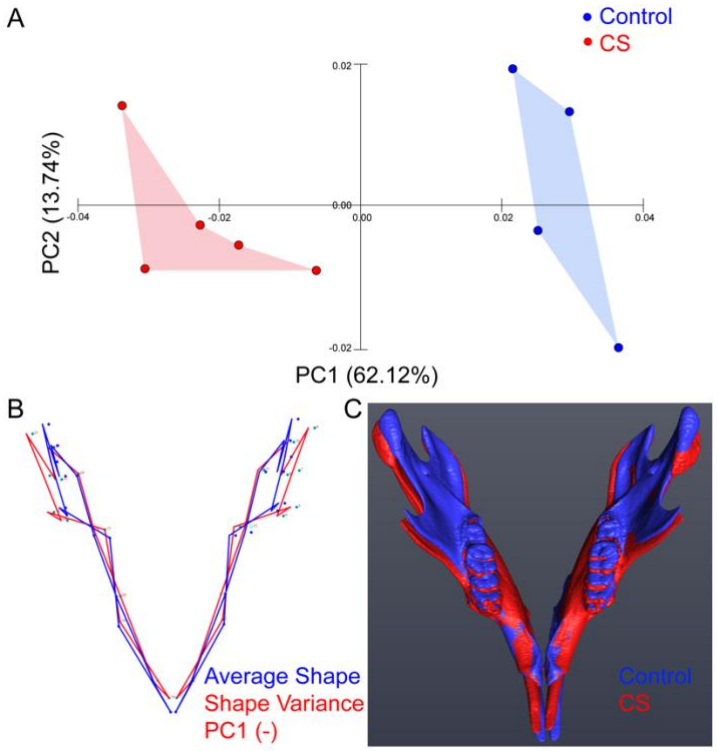


Figure 3. (A) PCA plot of CS and control mandibles. (B) Wire frame diagrams of the average mandible shape (blue) and the morph of negative PC1 (red), and (C) superimposition of a control (blue) and CS (red) mandibles, showing the primary shape difference in the mandible between control and CS is the widening of the condyles laterally.

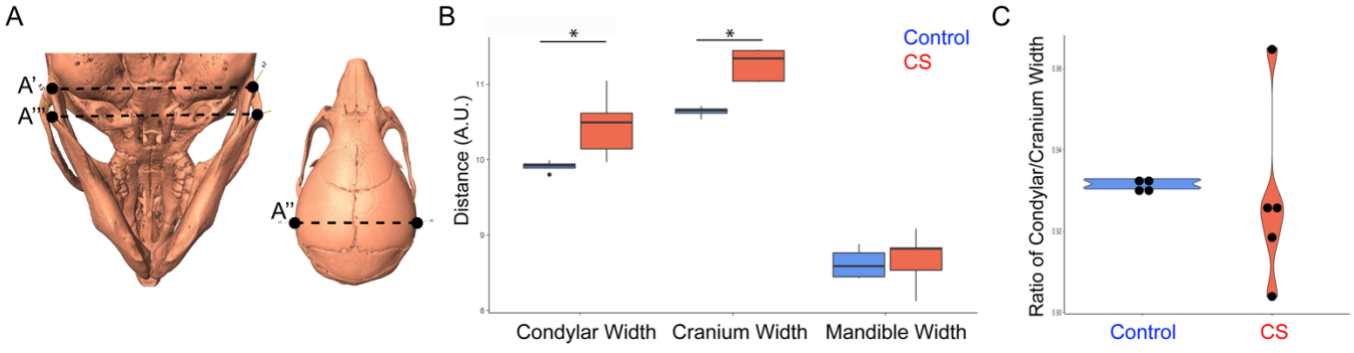


Figure 4. (A) Images showing the measurements of condylar width (A'), cranium width (A"), and mandible width (A"'). (B) Graph of the measurements show increased condylar and cranium width in CS compared to control, and no difference in mandible width. (C) Violin plot showing no significant difference in the ratio of condylar and cranium width in CS compared to control, and so the increase in condyle width coordinates with the increase in cranium width in CS. (*p<0.05)

**Aim 2: Utilize CS (*HRas*$^{G12V}$) mice to understand the role of Ras signaling in development and growth of the TMJ**

In order to further understand the malocclusion in CS, we analyzed the CS mouse model, performing geometric morphometric and histologic analysis. To analyze the shape of the CS mandible, we collected the skulls of postnatal day (P) 21 CS (N=5) and control (N=4) mice and performed μCT. The isosurfaces of the mandibles were generated in Avizo, and the entire mandible and condyles were landmarked and morphometric analysis was completed. The shape of the CS mandibles differed significantly from control, and the most significant difference, principle component (PC) 1 accounting for 62.12% of the variation, was the distance between the condyles (Figure 3). **The condyles are wider and curved laterally in the CS mice compared to control.** While skull width (measured at the widest portion of the parietal bone) and condylar width (measured from the most lateral aspect of the condylar head) is significantly increased in CS compared to control, the mandible width (measured from the lateral aspect of the angular process) does not differ significantly (Figure 4 A,B). There is no significant difference between the ratio of condyle width to the cranium width in CS compared to control (Figure 4 C). Thus, **the widening between the condyles coordinates with the widening of the cranium in CS mice** and suggests that the changes in the mandible shape may be secondary to development of the skull.

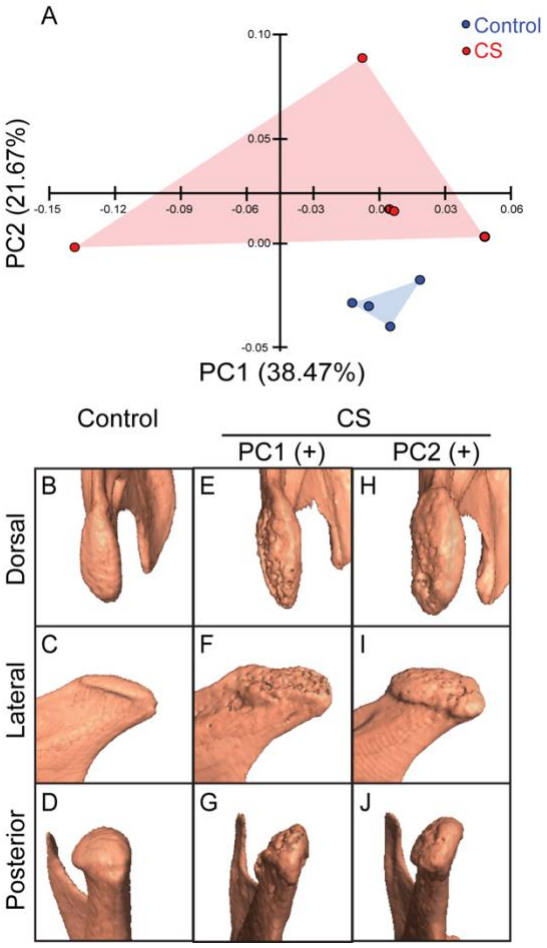


Figure 5. (A) PCA plot shows no significant difference in right condylar head shape between control and CS. (B-J) Representative images of narrowing in the medio-lateral (E-G) and antero-posterior (H-J) dimensions and pitting (E-J) in CS condyles compared to control (B-D).

We also analyzed the condylar heads using semi-landmarking in CS and control mice, which provides more precise shape changes in the condylar surface compared to morphometric analysis of the entire mandible (Hassan et. al., Ortho. & Cranio. Res., 2018). Principle components analysis (PCA) shows that overall, the shape of the CS condyles did not differ significantly from control; however, there was a great deal of variation in the CS condylar head shape, and the CS condylar heads were narrower medio-laterally and antero-posteriorly towards the positive end of PC1 and PC2, respectively (Figure 5). In addition, **there was pitting and erosion on the surface of the CS condyles that was not observed in controls** (Figure 5). This pitting may suggest

erosion due to wear between the condyle and glenoid fossa postnatally, with use of the joint, or possibly an adaptation of the condyle to the widened skull in CS.

In order to test the hypothesis that changes in the shape of the mandible and condyle are secondary to the growth of the skull in CS, during the PFA, **I generated a transgenic Cre mouse line with Cre expression driven by the pharyngeal enhancer of *Hand2* specifically in the mandible.** The *Hand2*$^{Cre}$ is expressed as early as E10.5 in pharyngeal arches 1 and 2, and by E14.5, its expression is specific to the mandible (Figure 6). I am currently characterizing the *Hand2*$^{Cre}$ mouse and generating *Hras*$^{G12V}$;*Hand2*$^{Cre}$ mice to test the role of Ras in the mandible. I hypothesize that if the shape changes are not intrinsic to the mandible but secondary to the skull defects in the CS mouse model, then the *Hras*$^{G12V}$;*Hand2*$^{Cre}$ mouse will have no mandibular phenotype or class III malocclusion. In addition, this mouse will be useful in my future projects studying the TMJ and for the field in general since **this Cre line is the first with specific mandibular expression**.

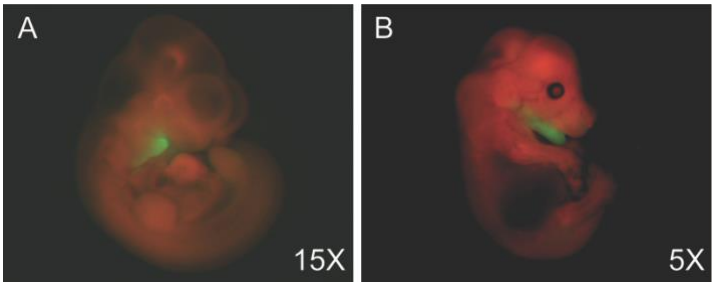


Figure 6. Images of *Hand2*$^{Cre}$;*R26R*$^{mTmG}$ embryos showing GFP expression (green) driven by Cre recombination specifically in pharyngeal arches 1 and 2 at E10.5 (A) and in the mandible at E14.5 (B).

Since the pitting phenotype on the condylar surface was striking, we decided to further measure the bone density of the condyle, mandible, and skull in the CS mouse. Although CS individuals have been found to have decreased bone mineral density (Leoni et. al., Mol. Genet. Metab., 2014), bone density has not been investigated in the CS mouse. We found that overall, **CS mice have a significantly decreased relative bone density compared to control mice, and the decrease was most significant in the condyle (20%) compared to the mandible (10%) and skull (8%)** (Figure 7).

In order to further understand the condylar changes at the tissue

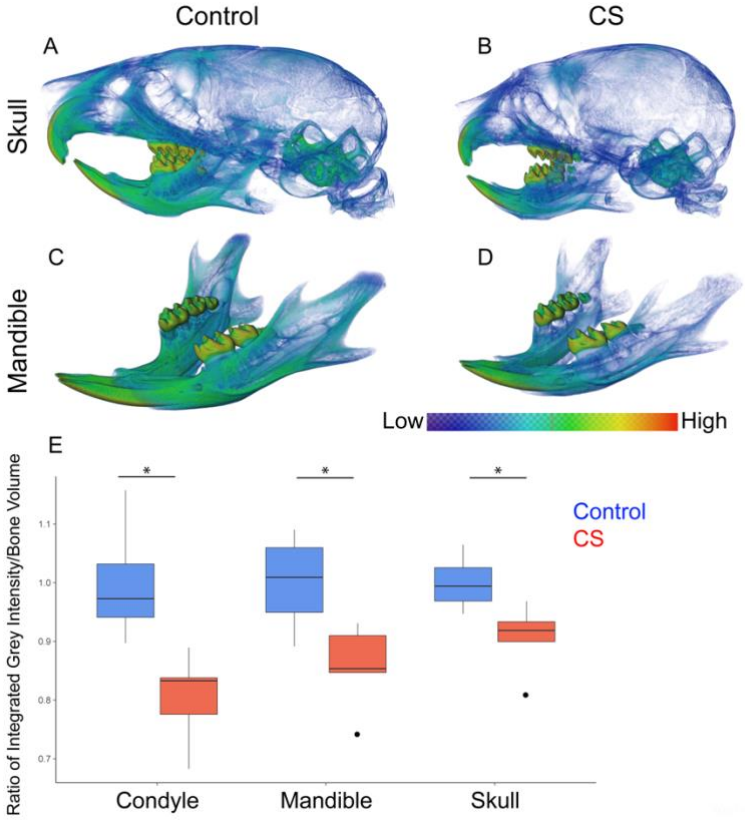


Figure 7. Reconstructions showing relative bone density of control (A,C) and CS (B,D) skulls and mandibles. Blue is less dense and red is more dense. (E) Graph of the ratio of relative bone density to bone volume shows significantly decreased bone density in the condyle, mandible, and skull in CS compared to control.

and cellular level, we next completed histological analysis of the TMJ in CS and control mice. H&E staining showed that at p4, the morphology of the CS TMJ was indistinguishable from control (N=3, Figure 8, A,B), suggesting that the development of the mandible and TMJ is normal in the CS mice. However, at p21, **the condylar head appeared flattened with decreased height, and the mandibular condylar cartilage (MCC) was thinner and contained fewer hypertrophic chondroblasts in the CS mouse compared to control** (N=3, Figure 8).

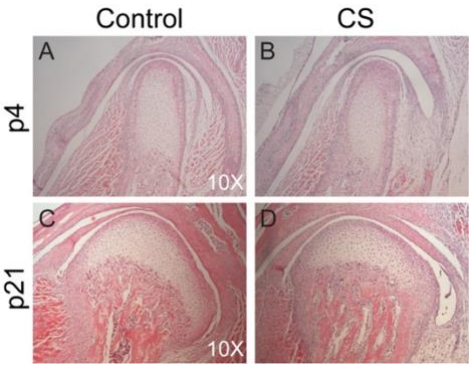


Figure 8. (A-D) Images of H&E stained coronal sections of the TMJ in control and CS mice. Note the TMJ is indistinguishable in control (A) and CS (B) at p4, and the condylar head is shorter in height, and MCC is thinner in the CS condyle (D) compared to control (C) at p21.

In order to further analyze the change in cellular composition of the MCC, we completed RNA scope on p4 mice with probes against *Sox9*, *Col2*, *Col10*, and *Col1*. We found no difference in expression of *Sox9* or *Col1*, however, there was decreased expression of *Col2* and *Col10* in the CS mice compared to control (N=3, Figure 9), which are expressed in the maturation and hypertrophic chondrocytes, respectively. These data suggest a **decrease in proliferative and hypertrophic chondroblasts in the CS MCC.** The flattening of the condylar head and thinning of the MCC in CS mice correlates with the wear on the condylar heads observed in the μCT data and suggests adaptive changes at the condyle postnatally.

Since our data suggest the mandibular size and shape is not the primary contributing factor to the class III malocclusion in CS, we have also further characterized the maxillary contribution. Our preliminary data showed there is a decrease in length of the cranial base and maxilla and early fusion of the intersphenoid synchondrosis (ISS) in the CS mouse compared to control. In order to determine when the early fusion of the ISS occurs and the changes in the morphology of the ISS, we collected additional CS and control heads at p5 and p10 for histologic analysis. At p5, the ISS is still patent in CS and control mice, with chondrocytes filling the synchondrosis (Figure 10). At p5, the reserve, proliferative, and hypertrophic chondrocyte zones in the ISS are proportional in the control, and while the zones are present in the CS ISS, the overall chondrocyte zone is narrower in the CS mice compared to control at p5 (Figure 10 A,B). By p10, the chondrocyte zones are present and in equal proportions in the control, however, in the

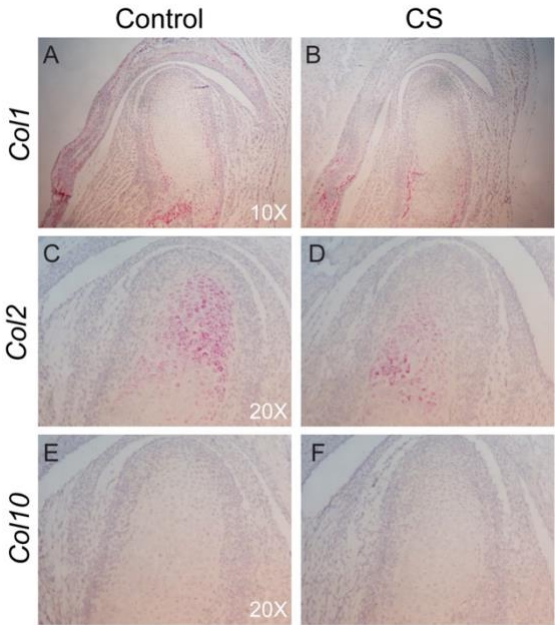


Figure 9. (A-F) RNA Scope on coronal TMJ sections shows no significant difference in expression (red dots) of *Col1* (A,B) and decrease in expression of *Col2* (C,D) and *Col10* (E,F) in CS compared to control at p4.

CS ISS, the chondrocyte zone has been filled with endochondral bone and is completely fused (Figure 10 C,D). Thus, **early fusion of the ISS occurs by p10**, which may contribute to the maxillary hypoplasia phenotype in CS, and the maxillary phenotype is being actively investigated by members of the Klein Lab.

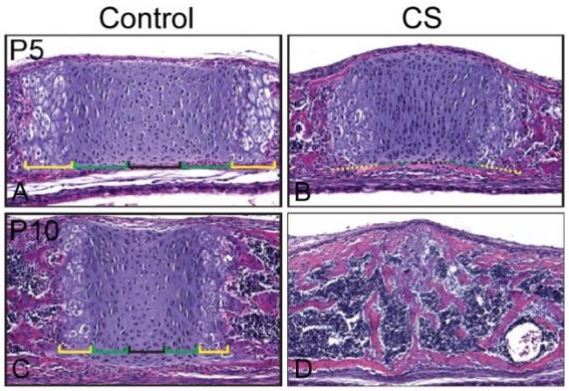


Figure 10. (A-D) Images of H&E stained sagittal sections of the ISS show that at P5, the synchondrosis is patent and contains the reserve (black bar), proliferative (green bar), and hypertrophic (yellow bar) chondrocyte zones in the control and CS mice, however, it is narrower in CS (B) than control (A). By p10, the ISS is patent with chondrocyte zones present in control (C) while the ISS is fused and filled with bone in the CS mice (D).

## CONCLUSIONS

Individuals with CS have a higher incidence of class III malocclusion, which in our cohort, is skeletal in nature and primarily due to a hypoplastic maxilla. In the CS mouse, we found that the major difference in the shape of the CS mandibles was an increase in the width of the condyles which matched the increased width of the skull. In addition, there was narrowing in the medio-lateral and antero-posterior widths of the condylar heads. There was also erosion and pitting on the CS condylar heads, and the relative mineral bone density of the CS mandible, condyle, and skull was decreased compared to control. Finally, the mandibular condylar cartilage was thinner with decreased proliferative and hypertrophic chondrocytes. These data suggest the development of the mandible is normal in CS; however, the mandible adapts and changes shape secondarily to widening of the skull and shortening of the maxilla and cranial base in CS, although additional work is necessary to test this hypothesis. Overall, this project shed light on the role of Ras signaling in the development and growth of the mandible and maintenance of the MCC.

The goals of the **EDUCATIONAL**, **TEACHING SKILLS**, and **CLINICAL SKILLS PLANS** were followed closely and achieved during the PFA award period. Overall, the skills obtained by adhering to these plans with the guidance of mentors helped me successfully obtain a NIDCR K08 award which began July 1, 2018 and a tenure-track, assistant professor position in the UCSF School of Dentistry, Department of Orofacial Sciences, Division of Craniofacial Anomalies which began January 1, 2018. Please find the goals achieved for the PFA development plan detailed below.

The goals of the **EDUCATIONAL PLAN** were to: (1) expand my knowledge in developmental and craniofacial biology, particularly TMJ, (2) learn new technologies and techniques, and (3) develop faculty skills including communication and grant and manuscript writing. During the AAOF PFA award period, I made progress in these three areas and accomplished many of the goals with the help of my mentors as outlined below.

During the award period, I was able to increase my knowledge in the craniofacial biology and TMJ fields by attending seminars at UCSF including the twice monthly Program in Craniofacial Biology (PCB) seminar series and the weekly Klein lab meetings, as well as monthly small group meetings. I also spent a great deal of time reading and learning about TMJ development and pathologies associated with this joint, and meeting and networking with people in the field at conferences, which has enabled me to develop ideas and hypotheses upon which I am building my independent research program.

During the PFA, I dedicated 60% of my time in the lab, completing the proposed experiments and learning new techniques such as Micro-CT analysis and TMJ histology. I was able to obtain technical advice and help interpreting data during meetings with both of my mentors, Dr. Ophir Klein and Dr. Jeffrey Bush, every other week. I also learned how to perform morphometric analysis with Dr. Marcucio and with additional assistance from members of the Klein Lab and Jheon Lab at UCSF.

During the fellowship, I had the opportunity to present twice per year at the Klein Lab Meeting and once per year at the PCB research in progress seminar, which provided excellent opportunities to receive feedback on the presentation, data, and project directions. I attended and presented at national meetings including the Consortium for Orthodontic Advances in Science & Technology (COAST) meeting at which I gave an oral general session presentation and Society for Developmental Biology (West Coast Regional) meeting where I presented a poster. I completed the Grant Writing Workshop online course offered by the UCSF Training In Clinical Research (TICR) program and submitted and successfully obtained a K08 from NIDCR, which will provide salary and lab support for the next 5 years.

The goals of the **TEACHING SKILLS PLAN** during the AAOF PFA were to further develop my teaching skills by presenting didactic lectures to pre-doctoral students and orthodontic residents (10% of my time) and receive feedback and mentorship from Dr. Gerald Nelson. During the AAOF fellowship, I had the opportunity to teach both predoctoral students and orthodontic residents. I gave talks on my research and lectured for the PCC 134 course on Development and etiology of malocclusion and Surgical Orthodontics to the predoc students. I participated in the First Year Resident Orientation, giving lectures and leading preclinical lab sessions, and gave talks each year on my research in the Orthodontic Research Seminar (OT461). The goals of the teaching skills plan were achieved, and the support of the AAOF allowed me to hone teaching skills that

I am currently using in my faculty position as I take on additional teaching responsibilities for both predoc students and orthodontic residents.

The goals of the **CLINICAL SKILLS PLAN** were to continue to develop my skills as an orthodontist by practicing 8 hours per week (20% of my time). During the AAOF PFA, I obtained a one day per week associate position with Dr. Yan Kalika in his office, Image Orthodontics. At this downtown San Francisco office, I treated adults and children, including phase I and surgical cases, with many different approaches, such as clear aligner therapy (Invisalign) and virtual orthodontic treatment planning (Suresmile). I received a great deal of mentorship from Dr. Kalika, and in the associate position, for new patients, I proposed treatment plans which he would review and provide feedback, and I was also able to ask him questions about mechanics and patient management during treatment. During the AAOF PFA, I also prepared and presented cases for the American Board of Orthodontics Clinical Exam, and I was Board certified in October 2018. Thus, I gained a great deal of experience in my clinical practice during the AAOF PFA, and I am continuing to develop as a clinician in my faculty position, caring for individuals with craniofacial anomalies at the UCSF Craniofacial Center one day per week, and treating patients at the UCSF Orthodontic Faculty Practice one half day per week.

Thus, the goals of the PFA development plan were achieved, and the AAOF PFA was instrumental in advancing my career. The AAOF PFA provided resources for my postdoctoral training to both continue to develop my research project and practice clinical orthodontics which has allowed me to secure additional NIH funding and a faculty position, and so I am very grateful for the support of the AAOF PFA.