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CONFERENCE REPORT

The Society for Craniofacial Genetics and Developmental Biology 44th Annual Meeting

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Abstract

The Society for Craniofacial Genetics and Developmental Biology (SCGDB) held its 44th Annual Meeting in a virtual format on October 18–19, 2021. The SCGDB meeting included presentation of the SCGDB Distinguished Scientists in Craniofacial Research Awards to Drs. Paul Trainor and Jeff Bush and four scientific sessions on the genomics of craniofacial development, craniofacial morphogenesis and regeneration, translational craniofacial biology and signaling during craniofacial development. The meeting also included workshops on professional development for faculty and trainees, National Institutes of Health (NIH)/National Institute of Craniofacial and Dental Research funding and usage of Genomics Software, as well as two poster sessions. An exhibitor booth run by FaceBase was also present to facilitate the upload and download of datasets relevant to the craniofacial community. Over 200 attendees from 12 countries and 23 states, representing over 80 different scientific institutions, participated. This diverse group of scientists included cell biologists, developmental biologists, and clinical geneticists. Although the continuing COVID-19 pandemic forced a virtual meeting format for a second year in a row, the meeting platform provided ample opportunities for participant interactions and discussions, thus strengthening the community.

1 | PROFESSIONAL DEVELOPMENT WORKSHOPS

Professional Development Workshop for Faculty

Chair: Dr. David Clouthier, Society for Craniofacial Genetics and Developmental Biology **(SCGDB) President (University of Colorado AMC)**

Dr. Laura Johnson (University of Missouri) and **Dr. Alexandra Wink (University of Massachusetts)** presented a workshop entitled "How to Make the Most of Your First Five Years as Faculty." Dr. Johnson, Chair of the American Association of Anatomy (AAA) Professional Development Committee, and Dr. Wink, a member of the AAA Professional Development Committee, are recognized experts in scientific career development. This workshop was designed to give participants a framework to navigate the first 5 years of their permanent faculty position. Drs. Johnson and Wink first described the main objective of a new faculty member, which is to build their career in order to be promoted on time. The presenters then shared strategies that have helped them work toward this objective, including software tools for daily and project organization that are freely available. This interactive session included opportunities to reflect on and practice developing one's career mission statement and goals. These activities were designed to help young faculty develop their professional identity, think strategically about prioritizing tasks, and selecting mentors.

Professional Development Workshop for Graduate Students and Postdocs

Chair: Dr. Amy Merrill, SCGDB Vice President (University of Southern California)

Dr. Aidan Ruth (Saint Louis University School of Medicine) and **Dr. Mikaela Stiver (McGill University)** presented a hands-on professional development workshop for graduate students and postdoctoral fellows. Drs. Ruth and Stiver are also members of the AAA Professional Development Committee and experts in scientific career development. First, they discussed the seven habits of highly effective graduate students and postdocs, which are to read and write every day, build relationships, ask for help, embrace mistakes, maintain a healthy balance, stay organized, and proactively make plans. Next, they led an interactive goal-setting exercise, where participants practiced a three-step process of setting professional and personal goals, breaking down their goals into manageable steps, and recognizing potential barriers to achieving these goals and identifying potential solutions. This workshop gave participants concrete tools and strategies to successfully meet their objectives in graduate school and beyond.

National Institute of Craniofacial and Dental Research (NIDCR) Lunchtime Workshop

Chair: Dr. Amy Merrill, SCGDB Vice President (University of Southern California)

Dr. Leslie Frieden, the Acting Chief of the Research Training and Career Development Branch, and **Dr. Katherine Stein**, the Director of the Developmental Biology and Genetics Program (Division of Extramural Research), led a workshop focused on NIDCR funding. They discussed the phases of the grant application life cycle and specific mechanisms for funding that included research grant programs, career development awards, research training and fellowship awards, and competitive funding opportunities to enhance diversity of the research workforce in dental, oral, and craniofacial research. Their presentation also highlighted NIDCR's research priorities and new funding opportunities for both trainees and faculty, and concluded with an open question-and-answer session. Further questions about NIDCR-related funding topics can be addressed to Drs. Frieden (leslie.frieden@nih.gov) and Stein (kathryn.stein@nih.gov).

2 | GENOMIC SOFTWARE WORKSHOP

Chair: Dr. Samantha Brugmann, SCGDB Secretary (Cincinnati Children's Hospital Medical Center)

Dr. Nicole Weaver, a clinical geneticist from Cincinnati Children's Hospital Medical Center, presented the third workshop of the meeting. The advent of rapidly advancing technologies that allow us to sequence patients and identify variants causal for human disease, coupled with the increased desired for "translational" research, results in a tremendous need to keep the basic science community abreast of user-friendly tools and resources. Dr. Weaver meticulously laid out the current issues that impact the field including bottlenecks of the diagnostic odyssey and examples for what geneticist look for to make a confident diagnosis. Throughout the workshop, Dr. Weaver walked the audience through the use and limitations of several online resources including gnomAD, VarSome, PolyPhen2, and GeneMatcher. A lively Q&A session immediately followed this informative workshop. In summary, Dr. Weaver provided key take home messages including the understanding that exome/genome data will not always provide an answer for patient diagnosis and that communication between clinicians and researchers will remain essential for functional validation of candidate variants. Slides from Dr. Weaver's talk are posted on the SCGDB website (https://www.scgdb.org/new-page-past-meet).

3 | SCGDB DISTINGUISHED SCIENTISTS IN CRANIOFACIAL RESEARCH AWARDS

Chair: Dr. David Clouthier, SCGDB President (University of Colorado)

The SCGDB presented awards to members for their contributions to the Society: the David Bixler Distinguished Scientist Award, and the Marylou Buyse Distinguished Scientist Award.

The **David Bixler Distinguished Scientist Award** is named in honor of the achievements of the Society's first President, Dr. David Bixler, a pioneer in genotyping craniofacial syndromes. This award recognizes a senior scientist who has provided long-term distinguished leadership to the SCGDB and has made meritorious contributions to the field of craniofacial sciences. The recipient of this year's award was **Dr. Paul Trainor**, who is an Investigator at the Stowers Institute for Medical Research in Kansas City, MO. Among other roles, Dr. Trainor is also Editor-in-Chief of Developmental Dynamics. Dr. Trainor received his BSc and PhD from Children's Medical Research Institute at the University of Sydney (Australia) under the guidance of Dr. Patrick Tam. This was followed by a postdoctoral research fellowship at the National Institute for Medical Research in London (UK) with Dr. Robb Krumlauf. Dr. Trainor's lab focuses on understanding neural crest cells and craniofacial development, concentrating on mechanisms that regulate neural crest cell formation, migration, and differentiation. As part of these interests, he has had success in relating changes observed in animal models to disease mechanisms in human craniofacial syndromes. He has also made significant contributions to the SCGDB by serving as Vice-President, President, and Past-President, during which he transformed the SCGDB into a financially sound society attracting speakers and attendees from around the world to the annual meeting.

Dr. Trainor began his talk by describing his lab's investigation into the molecular and cellular basis of Treacher Collins syndrome (TCS), a disorder characterized by hypoplasia of the zygomatic complex and conductive hearing loss. The first gene identified to be causative in TCS was *TCOF1*. Using *Tcof1*$^{-/-}$ mice created by Mike and Jill Dixon, the Trainor lab found mutant mice had defects resembling TCS that were consistent with aberrant neural crest cell development. *TCOF1* encodes Treacle, a protein associated with the nucleolus (along with Polr1a, Polr1c and Polr1d), suggesting a role in ribosomal DNA (rDNA) transcription and ribosome biogenesis. Treacle is also associated with DNA damage repair through its association with the MRN complex (composed of Mre11, Rad50 and Nbs1). Using mouse and zebrafish knockouts of *Tcof1*, *Polr1a*, *Polr1c*, and *Polr1d*, Dr. Trainor showed that

neural crest cell development in mutants was disrupted and accompanied by an increase in both cell cycle arrest and apoptosis. This occurred due to downregulation of rDNA transcription and, subsequently, loss of ribosomal biogenesis and stabilization of p53, which lead to increased cell death of neural crest cells. The Trainor lab is now looking at the events in the different centers of the nucleolus in mutants. Through labeling of the different centers with specific markers, it appears that inhibition of Pol1 causes a collapse of the nucleolus; in cells that make it through mitosis, the nucleolus does not reform.

Dr. Trainor then described his lab's work in understanding why the craniofacial complex appears so sensitive to disruption of the Tcof/Polr complex. During neural crest cell specification, large numbers of neural crest cells are made, with this process requiring extensive protein production that relies on increased rDNA transcription and protein translation. Single-cell RNA sequencing showed that while genes encoding Pol1 units are expressed throughout the head region, they are highly expressed in both the neuroepithelium and neural crest cells. The Trainor lab has previously shown that p53 inhibition prevents apoptosis of neural crest cells in the neuroepithelium and thus prevents TCF. The protection, thus, likely arises from preventing early neural crest cell apoptosis prior to migration. Dr. Trainor finished by describing his lab's recent work illustrating that Treacle works with the MRN complex to protect the neuroepithelium from oxidative stress and damage. Breeding *Tcof1*$^{-/-}$ mice onto different genetic backgrounds illustrated that mouse strains with higher oxidative stress markers had increased facial defects. These findings thus suggest that gene–environmental interactions likely contribute to the phenotypic variability of TCS.

The **Marylou Buyse Distinguished Scientist Award** was created by the SCGDB to honor the memory of the Society's first female President, Dr. Marylou Buyse, a prominent clinical geneticist. This award recognizes a mid-career scientist who has made important contributions to the craniofacial sciences. The recipient of this award was **Dr. Jeffery O. Bush**, Professor of Cell and Tissue Biology in the School of Dentistry at the University of California San Francisco. Dr. Bush received his PhD in Developmental Biology and Genetics from Dr. Rulang Jiang's lab at the University of Rochester. In 2005, Dr. Bush started a postdoctoral fellowship with Dr. Phillipe Soriano, first at the Fred Hutchinson Cancer Research Center in Seattle and then at the Mt. Sinai School of Medicine when Dr. Soriano moved his lab. In 2011, Dr. Bush started his position at the University of California San Francisco as an Assistant Professor and has quickly moved up the ranks, being named Associate Professor in 2017 and Professor in 2021. His lab uses a battery of approaches based in mouse molecular genetics combined with ex vivo culture, live imaging, patient-specific induced pluripotent stem cell (iPSC) modeling, and biophysical methods, to understand how signaling regulates cellular organization and tissue shape in craniofacial development and disease.

Dr. Bush first discussed his lab's work to understand how the midline epithelial seam (MES) is removed during palatal fusion, a long-standing question whose answer has remained elusive. Using live imaging, the Bush lab was able to show that epithelial cells of each palatal shelf moved toward the midline by mediolateral convergence to form a shared seam. The epithelial cells of the seam reorganized by coalescing into islands and trails that underwent collective migration toward the oral and nasal epithelial surface, where many of them were ultimately extruded into the oral and nasal cavity. As previously reported, extensive apoptosis was also observed throughout the MES at every stage of the process. Thus, fusion of the MES involves directed migration, apoptosis, and extrusion of MES cells.

Dr. Bush next discussed his lab's investigation into how cell death occurs in MES cells. Cell death can occur though both extrinsic and intrinsic mechanisms, with the intrinsic pathway activating Bax and Bak. These two proteins are required for mitochondrial permeabilization and formation of the apoptosome, which activates caspases. Dr. Bush showed that conditional deletion of *Bax* and *Bak* in the ectoderm blocked apoptosis of the islands and trails of MES cells, though palate fusion still occurred through collective migration. This suggested that apoptosis or extrusion of MES cells can be overcome for palatal shelve fusion. Finally, Dr. Bush showed that migration of MES cells required nonmuscle myosin II, a common mechanism for force generation during morphogenesis of other tissues. Conditional inactivation of *Myh9* (which encodes nonmuscle myosin heavy chain IIA) in the ectoderm blocked migration of MES cells and led to loss of their collective behavior. This may partially explain the association of *MYH9* with cleft lip with or without cleft palate. Dr. Bush closed by showing live imaging of fusion between the lateral and medial nasal prominences and early analysis that suggested a role for actomyosin contractility in that process as well.

SCIENTIFIC SESSION I: Genomics of Craniofacial Development

Chair: Dr. Rolf Stottmann (Nationwide Children's Hospital)

Dr. Donna Martin (University of Michigan) was the keynote speaker for the first session focused on genomic approaches to studying craniofacial development. The Martin lab has a long history of studying the role of the ATP-dependent chromatin remodeler, *chromodomain helicase DNA binding protein 7* (*CHD7*) in CHARGE syndrome. CHARGE syndrome occurs in approximately 1 in 10,000 births and is a genetic syndrome with a consistent pattern of congenital differences including *C*oloboma of the eye, *H*eart defects, *A*tresia of the choanae, *R*estriction of *G*rowth, and *E*ar abnormalities and deafness. This is a disorder of sensory deprivation, and one well-known cause is autosomal dominant haploinsufficiency of *CHD7*. Despite a body-wide requirement for proper chromatin structure, *CHD7* is not ubiquitously expressed and thus CHARGE syndrome phenotypes are restricted to the organs expressing *CHD7*. Dr. Martin's lab has invested significant effort in studying the tissue specific roles of *Chd7* in mouse models taking advantage of a conditional allele of *Chd7* and the Cre-Lox system. In this talk, Dr. Martin focused on recent work highlighting the role of *Chd7* in ear development. An elegant method of filling the inner ear with paint was utilized to highlight morphogenesis abnormalities in different genetic ablation paradigms. Ablation of *Chd7* from the otic mesenchyme using *T-Cre* did not result in significant phenotypes. Using a *Pax2-Cre* to remove *Chd7* function in the otocyst resulted in a reduction in the semi-circular canals and a hypoplastic cochlea. Subsequent examination of the cochlear sensory epithelium showed disrupted neurite projections in spiral ganglion neurons and

an increase in outer hair cells in the apex of the cochlea, but not at the base. The hair cell defect was further explored by removing *Chd7* in these cells alone with *Atoh1-Cre*, which did not recapitulate the phenotypes seen in the otocyst ablation. Together, these data suggested that cochlear phenotypes are not simply a cell autonomous effect upon loss of *Chd7*. This discussion highlighted the power of mouse genetic tools to perform careful genetic dissection of tissue-specific requirements for important developmental genes. In this way, one can generate insights into specific disease mechanisms across development even when the human syndromes can collectively affect a number of different organs with wide-ranging effects.

Next, **Dr. Marco Trizzino (Thomas Jefferson University)** spoke about the role of the *AT rich interactive domain 1A/1B (ARID1A* and *ARID1B*) genes in regulating neural crest cell pluripotency and differentiation. These proteins are mutually exclusive subunits of BAF chromatin remodeling complexes, which are critical components of the cell machinery regulating nucleosome positioning and chromatin accessibility. ARID1A is associated with an embryonic stem cell BAF signature to control pluripotency and self-renewal. De novo variants in both *ARID1A* and *ARID1B* are known to cause Coffin–Siris syndrome, a disorder characterized by both neurological and craniofacial features. ARID1B is also associated with other neurodevelopmental disorders. Dr. Trizzino presented work in iPSCs derived from control and *ARID1B* heterozygous Coffin–Siris patients that were differentiated into neural crest cells. ARID1B regulates the exit of neural crest cells from pluripotency, with maturing neural crest cells normally down-regulating ARID1A in favor of ARID1B. Coffin–Siris patient-derived cells show reduced potential for differentiation into neural crest cells and a concomitant gain of chromatin accessibility at approximately 5500 genomic regions as compared to control cells, thus showing very broad effects of improper regulation of the ARID1A/ARID1B proteins in this differentiation process. Approximately, 90% of regions with increased chromatin accessibility were identified as enhancers and differential expression analysis indicated approximately 2400 differentially expressed genes between patient and control cells. Pathway analysis of these genes showed a reduction in pluripotency pathway genes. These studies further elucidated the consequences for ARID1A/ARID1B dysregulation and provided a potential molecular mechanism for Coffin–Siris syndrome.

Next, **Dr. Casey Griffin (New York University)** presented recent work on the role of *Splicing factor 3b subunit 2* (*SF3B2*) in craniofacial microsomia. Craniofacial microsomia represents a relatively common spectrum of craniofacial conditions that frequently presents with auricular malformations and mandibular hypoplasia. The identity of tissues affected in craniofacial microsomia suggests that a deficit of neural crest cells may be at least partially responsible for associated phenotypes. SF3B2 is a protein in the spliceosome complex important for the processing of pre-mRNA to mRNA. Interestingly SF3B2 interacts directly with SF3B4, another component of the spliceosome associated with another craniofacial condition, Nager syndrome. Depletion of SF3B2 in *Xenopus* embryos was used to address the hypothesis that reduced expression of this gene could lead to the craniofacial malformations through an effect on neural crest cell production and/or survival. SF3B2 knockdown did indeed lead to reduced expression of several neural crest genes (*sox10*, *snai2*, *tfap2e*) and increased expression of neural plate genes (*sox2*). Later in development, SF3B2-depleted tadpoles had defective or missing neural crest-derived craniofacial cartilages. These studies supported the conclusion that a reduced neural crest cell population was a key contributor to craniofacial microsomia, with reduced SF3B2 function and subsequent spliceosome disruption as a likely molecular mechanism.

The session concluded with a talk from **Andrea Wilderman (University of Connecticut)** who is interested in studying noncoding regions of the genome with elements likely to contain features contributing to gene regulation for proper craniofacial development. These regulatory regions are a high priority for identification of putative noncoding variants leading to congenital craniofacial malformations. The lab in which she is a postdoc has done impressive work and described the identification of putative craniofacial "super enhancer" regions. These noncoding regions, also called locus control regions, coordinately activated enhancers poised to play large roles in regulating tissue development. Ms. Wilderman discussed analysis of a specific enhancer with many interesting potential regulatory features, but no genes within a reasonable genomic distance known to be important for craniofacial development. A three-dimensional chromatin structural analysis, however, indicated possible interactions with the HoxA gene cluster. The role of this super enhancer was directly tested in mice by genetic deletion. Homozygous deletion in mice resulted in reduced viability and frequent orofacial clefts, phenocopying *Hoxa2*$^{-/-}$ mice. These results suggested that this was indeed a critical enhancer of the HoxA cluster and highlighted an attractive strategy to identify novel regulatory elements and potential variants within the noncoding genome. The speakers in this session highlighted how new strategies for analyzing the whole genome, together with classical experimental models (animal and cellular systems), could be utilized to gain a deeper understanding of both human development and disease.

SCIENTIFIC SESSION II: Craniofacial Morphogenesis and Regeneration

Chair: Jean-Pierre Saint-Jeannet, SCGDB Treasurer (New York University)

The second scientific session of the meeting focused on craniofacial morphogenesis and regeneration. The plenary speaker, **Dr. Karen Liu (King's College London)** started the session by summarizing work from her lab in the last decade investigating the cellular and molecular bases of craniosynostosis. Craniosynostosis is a birth defect occurring in approximately 1 in 2000 live births, where premature fusion of cranial bones interferes with skull and brain growth. Several years ago, Dr. Liu and colleagues proposed that the ciliopathy gene *Fuz* should be considered as a candidate craniosynostosis gene based on the observation that *Fuz* mouse mutant embryos had premature fusion of the coronal suture. Subsequent work from her lab revealed that these animals had an increased number of neural crest cells and that these cells were highly disorganized as they migrate. Later in development, mutant neural crest cells had a higher propensity to form mesenchymal condensations and made bone with increased mineralization. Altogether, these results indicated that *Fuz* is required at multiple

steps in the development of neural crest-derived skull bones. FUZ variants in humans have been associated with several anomalies including orofacial clefts, neural tube closure and heart defects. More recently a novel variant, FUZ p.R284P, was reported in monozygotic twins presenting with craniosynostosis. The Liu lab is currently investigating the function of the FUZ p.R284P variant by expressing the mutant protein in WT and *Fuz* mutant mouse embryonic fibroblasts. Interestingly, FUZ p.R284P behaves largely like the WT protein in these assays, promoting ciliogenesis and cilia-mediated Hedgehog signaling, thereby revealing cilia-dependent and independent functions of FUZ as the possible underlying cause of the skull ossification phenotype observed in patients expressing the FUZ p.R284P variant. Together, this work linking animal studies to human gene variants has the potential to provide novel information on the mechanisms underlying the phenotypic variations seen in human patients expressing distinct FUZ variants.

The next speaker, **Dr. Shannon Fisher (Boston University)** discussed recent work from her lab identifying risk loci for craniosynostosis. A genome-wide association study previously identified susceptibility loci for nonsyndromic sagittal craniosynostosis near BMP2 and BMPER, an extracellular BMP regulator that enhances BMP signaling in bone development. This observation led to the hypothesis that sequence variants in each enhancer region caused elevated expression of BMP2 or BMPER and were responsible for increased risk of craniosynostosis. To determine the mechanism of increased risk, Dr. Fisher and colleagues sought to identify the enhancers contained in the two risk loci and correlate sequence variants with functional changes. In these regions, they selected noncoding sequences based on conservation across species and tested their enhancer activity in transgenic zebrafish. They screened 55 sequences upstream of BMPER and found two enhancers active in early osteoblasts. The first one showed transgene expression in the osteogenic front at the growing edge of the frontal bone. The second one, which also contained a single-nucleotide polymorphism (SNP) associated with disease risk, was active early in a subset of craniofacial cartilages, and later the transgene was detected in osteoblasts of the frontal bone. They also tested 16 sequences from the risk locus downstream of BMP2 and found 2 enhancers active in early osteoblasts and cartilages, respectively. For each enhancer, they identified interactions with multiple transcription factors using a yeast one-hybrid assay. Ongoing experiments involve the generation of transgenic lines with variant enhancer sequences to assess whether altered activity and transcription factors binding may contribute to the increased risk of craniosynostosis.

Next, **Dr. Peter Fabian (University of Southern California)** presented work investigating the cellular diversity within the developing vertebrate head using single-cell RNA-Seq of the cranial neural crest lineage during the lifetime of zebrafish embryos. This analysis identified several skeletogenic progenitors, a number of respiratory cell types (typically found in the neural crest cell-derived gills) as well as a novel specialized dermal fibroblast cell population. These fibroblasts are characterized by the expression of genes encoding enzymes of the Phe/Tyr degradation pathway and are localized in the dermis between the skin and the developing cranial skeletal tissues. Mutations in enzymes of this pathway cause metabolic diseases in human. The most striking is alkaptonuria, also known as black bone disease, which is due to mutations in homogentisate 1,2-dioxygenase (HGD), the enzyme that converts homogentisate to 4-maleylacetoacetate in the tyrosine degradation pathway. In these patients, accumulation of homogentisate in the skin, bone, and cartilage results in blackening and brittleness of these tissues. To model this condition and understand the role of this novel population of dermal fibroblasts during craniofacial development, Dr. Fabian generated a zebrafish *hgd* mutant, which recapitulated the bone and cartilage blackening phenotype observed in human patients. Future studies will test the hypothesis that in addition to the well-documented function of the Phe/Tyr degradation pathway in the liver, the local degradation of Phe/Tyr metabolites by dermal fibroblasts plays an important protective role on the developing skeleton, presumably by buffering the excess of Phe/Tyr metabolites produced by skin melanocytes, which use Tyr to produce melanin.

For the last talk of the session, **Shawn Hallett (University of Michigan)** discussed experiments dissecting the contribution of parathyroid hormone related peptide (PTHrP)-expressing chondrocytes to the synchondrosis of the cranial base. PTHrP is a master regulator of endochondral bone growth. PTHrP deficient mice, first described in 1994, have shorter long bones and all chondrocyte layers of the growth plate are significantly shortened. These animals also have craniofacial defects, although the original report did not describe whether the cranial base was preferentially affected. Previous work in Noriaki Ono's lab reported that PTHrP+ cells in the resting zone of long bones are an important source of skeletal cells providing chondrocytes to all layers of the growth plate, including cells that can transdifferentiate into Col1a1+ osteoblasts. It is, however, unclear whether PTHrP+ skeletal stem cells exist in other endochondral bones such as the cranial base synchondroses. The cranial base is formed by endochondral ossification and contains growth plate cartilage, the spheno-occipital synchondrosis (SOS). Similar to the growth plate of long bones, the SOS is composed of layers of resting, proliferating and hypertrophic chondrocytes, though unlike the long bones, it has a bilateral organization converging toward an intervening central hypertrophic zone. To assess the differential roles of PTHrP+ cells in endochondral bone formation, Mr. Hallett compared PTHrP expression in the SOS and femoral growth plate using a *PTHrP-mCherry* knock-in allele. At P3, P6, and P9, PTHrP+ cells were sparsely detected throughout the SOS, while at P14, PTHrP+ cells were restricted to a wedge-shaped area on the lateral surface of the SOS. However, these cells did not label the central portion of the resting zone. Further lineage tracing analyses indicated that these PTHrP+ cells failed to differentiate and to contribute to the postnatal SOS. Therefore, in contrast to long bones, PTHrP+ chondrocytes in the SOS do not function as a source of skeletal stem cells. Future work will investigate the presence of skeletal stem cells in the synchondrosis using additional lineage tracing experiments and knockout mouse models. These findings point to important differences in the source of skeletal stem cells in endochondral bones of different origin.

SCIENTIFIC SESSION III: Translational Craniofacial Biology

Chair: Dr. Amy Merrill, SCGDB Vice President (University of Southern California)

Dr. Matthew Harris (Harvard University) started the session with a presentation on how his lab is merging comparative genomics with phylogenetic patterns, a strategy called "phylomapping," to identify pathways and genes underlying morphological complexity in the fish jaw. The Harris lab has a strong interest in the developmental logic that drives phenotypic diversity of craniofacial form. Dr. Harris described how his lab uses phylomapping to investigate the striking variation in jaw structure of Beloniformes fishes, which include halfbeaks, needlefishes, and flying fishes. Halfbeaks, which exhibit extension of the lower jaw, are relatively basal members of Beloniformes. Flying fishes and needlefishes, while nested within the halfbeak clades, have jaw morphologies distinct from that of halfbeaks: needlefishes have an elongated upper jaw, whereas flying fishes have lost the extended lower jaw. By comparing genomic samples from 43 species of Beloniformes, the Harris lab identified patterns of conserved and accelerated sequence evolution. Their analysis pinpointed coding variants that are fixed between members of the clade that share a particular jaw phenotype.

As proof of principle, Dr. Harris described examples of convergent exon variants that lead to amino acid substitution among needlefish species, which are located in or near genes with known roles in craniofacial development and disease. He predicted that these fixed variants could either change the function of their gene or, due to microsynteny, mark adjacent noncoding variants that are involved in transcriptional regulation of a neighboring gene. He then showed evidence supporting both of these predictions. The *agap1* locus, which contains a fixed coding variant within needlefishes, is associated with a significant risk variant for hemifacial microsomia in humans. While *agap1* is not predicted to be involved in craniofacial development, its gene neighbor *gbx2* is. Dr. Harris posited this fixed coding variant in *agap1* was a marker for an adjacent noncoding variant involved in regulating *gbx2*. Their analysis of noncoding sequences in *agap1* supported this idea. The *agap1* locus contains an ultra-conserved noncoding element in zebrafish (*agap1*:343bp) that is also found in the human *AGAP1* locus, which the Noonan group has shown contains a rapidly evolving human noncoding element that regulates spatiotemporal expression of *Gbx2* in the pharyngeal arch of humanized mice. Dr. Harris also discussed how *agap1*:343 bp was linked to loss of extended lower jaw in flying fishes. While this element was retained in needlefishes and halfbeaks, it contains a drastic deletion that included a key transcription factor binding site in flying fishes. Together, this strongly suggested that the *agap1*:343 bp element was directly related to lower jaw length and potentially represents a conserved regulatory hub for jaw size.

Dr. Harris concluded his talk with examples of fixed coding variants that lead to amino acid substitution among needlefishes that were expected to alter gene function. Phylomapping revealed a group of variants that resulted in a distinct pattern of convergent and covariant amino acid changes in a set of genes, including *piezo2* and *thbs2*, that were associated with craniofacial development and jaw length.

Dr. Jian Xu (University of Southern California) presented work from her group demonstrating that protein arginine methylation regulated osteoprogenitors during craniofacial development. The Xu lab has had a long-standing interested in the role of the protein arginine methyltransferases (Prmt) 1–9 in development and disease. Dr. Xu described how her lab used biochemistry and mouse genetics to identify a role for Prmt4 in calvaria osteogenesis. Dr. Xu's group found that *Prmt4*, the dominant Prmt expressed in osteogenic cells, was upregulated during osteoblast differentiation. Dr. Xu showed that Prmt4 methylates Runx2, a transcription factor critical for osteoblast differentiation, at four arginine residues within the transactivation domain. Next, she described that conditional loss of *Prmt4* in neural crest-derived osteoprogenitor cells with the *Wnt1-Cre* driver in mice caused failed closure of the posterior frontal suture, a phenotype resembling persistent anterior fontanels in the *RUNX2*-relate syndrome Cleidocranial Dysplasia. She then discussed possible cellular mechanisms responsible for the phenotype including proliferation, apoptosis, differentiation, and migration. While assays for proliferation, apoptosis, and differentiation suggested that differences in these processes were unlikely to account for the phenotype, DiI labeling in calvarial explants and in vitro cell migration assays indicated that Prmt4 was required for cell migration. Transcriptomic analysis of *Wnt1*-lineage cells in the frontal suture supported this idea: genes associated with matrix formation and cell migration were significantly downregulated in the *Prmt4* conditional knockout compared to control. Dr. Xu went on to show that loss of *Prmt4*, which was normally expressed in the osteogenic fronts of the frontal bones, led to reduced arginine methylation of Runx2 in vivo. She concluded her talk with evidence that Prmt4-mediated regulation of Runx2 altered its activity as a transcription factor. ChIP analysis revealed that loss of *Prmt4* reduced Runx2 occupancy at migratory genes. Together this study supported a model that Prmt4-mediated methylation of Runx2-regulated osteogenic cell migration during frontal suture development.

Next, **Dr. Soumya Rao (University of Missouri)** presented her work on the genetic basis of *O*culo-*A*uriculo-*V*ertebral *S*pectrum (OAVS). OAVS represents a phenotypically heterogenous condition that involves craniofacial differences including facial asymmetry, microtia, and ocular anomalies. Variants in the *MYT1* gene, which codes for a transcriptional suppressor of retinoic acid receptors, are associated with OAVS. This suggests that the retinoic acid pathway is involved in OAVS pathology. In support of the idea, Dr. Rao discussed how the spontaneous mouse line *small body and small ear* (*sbse*), which exhibited features of OAVS, harbored an inversion/deletion that elevated expression of two adjacent retinol dehydrogenase genes. Dr. Rao then described her search for novel OAVS genes using a cohort of nine isolated cases and three familial cases with autosomal dominant inheritance. Using whole-exome sequencing, she identified predicted loss-of-function variants in *SF3B2* in two families with mild OAVS phenotypes. SF3B2 is involved in pre-mRNA splicing as a component of the U2 snRNP splicing complex. Others have shown that haploinsufficiency of *SF3B2* gene caused OAVS and knockdown of *sf3b2* in *Xenopus* alters neural crest cell development. Therefore, Dr. Rao predicted that *SF3B2* was a major OAVS gene. Her collaboration with the Trainor lab showed that deletion of *sf3b2* in zebrafish induced cell cycle arrest and apoptosis of migratory cranial neural

crest cells to ultimately disrupt development of the eyes, ears, and pharyngeal arches. Dr. Rao went on to discuss how analysis of the OAVS cohort has also identified a single family with a predicted loss of function variant in splicing factor 1 (*SF1*), another gene involved in pre-mRNA splicing. Dr. Rao concluded her talk by describing de novo missense mutations in *SF3B2* in cases of OAVS with microtia and facial asymmetry. She predicted that the type of mutation in *SF3B2* likely explains phenotypic variability in OAVS.

The final talk of the session was presented by **Audrey Nickle (University of Southern California)**. Syndromic craniosynostosis, where fibrous joints between calvarial bones prematurely fuse, are commonly associated with variants in fibroblast growth factor receptor 2 (*FGFR2*). Ms. Nickle described how she is employing mouse genetics to reveal the mechanism of multi-suture craniosynostosis in the *FGFR2*-related syndrome Bent bone dysplasia syndrome (BBDS). BBDS, the most recently characterized *FGFR2* disorder, results from two dominant missense variants in the receptor's transmembrane domain. Ms. Nickle is leveraging a conditional knock-in allele that harbors the BBDS missense variant p.M391R ($Fgfr2^{M391R}$) to selectively activate the allele in neural crest- and mesoderm-derived mesenchyme that each make unique contributions to the calvarial bones and sutures. First, Ms. Nickle showed that neural crest-specific activation of $Fgfr2^{M391R}$ with *Wnt1-Cre* induced progressive multi-suture fusion during postnatal development. Interestingly, the sutures fused in this model included some where the bones were strictly mesoderm derived, suggesting that nonosteogenic cells from the neural crest lineage played a role in craniosynostosis. Lineage tracing experiments showed that coronal suture fusion originated from neural crest-derived cells that form ectopic bone within the nonosteogenic connective tissues, such as the dermis and dura. Next, Ms. Nickle revealed that mesoderm-specific activation of $Fgfr2^{M391R}$ with *Mesp1-Cre* only led to fusion of the mendosal suture in the posterior skull where there is little to no contribution from neural crest cells. Single-cell RNA-seq analyses further revealed that *Fgfr2* was expressed not only by osteogenic lineages, but also by suture-associated connective tissue. Finally, Ms. Nickle presented her progress in understanding the molecular mechanisms behind craniosynostosis in the BBDS mouse model. RNA-seq of the sutures indicated upregulation of the Wnt signaling pathway. All together her study revealed that activation of the BBDS variant in suture-associated connective tissue was linked with increased Wnt signaling and ectopic bone formation.

SCIENTIFIC SESSION IV: Signaling during Craniofacial Development

Chair: Dr. Samantha Brugmann, SCGDB Secretary (Cincinnati Children's Hospital Medical Center)

The fourth Scientific Session focused on discussing the signaling pathways that shape the craniofacial complex. **Dr. Gage Crump (University of Southern California)** kicked off the session by presenting work from his lab aimed at gaining a deeper understanding of the molecular factors that contribute to the diversification of cranial neural crest cells. As a multipotent cell population, neural crest cells generate a wide diversity of both nonectomesenchymal and ectomesenchymal derivatives, including most of connective and skeletal tissues of the vertebrate head. Yet how neural crest cells acquire such extraordinary lineage potential remains an unresolved question. To address this knowledge gap, Dr. Crump's lab focused on using single-cell technology. Single-cell genomic analysis provides several advantages over earlier approaches and can serve to help us understand the regulatory logic guiding neural crest cell lineage trajectories and connect embryonic patterning to neural crest cell fate acquisition.

Dr. Crump first focused on the use of single-cell analyses to aid in the discovery of novel neural crest cell-derived cell types. To profile transcriptomes and chromatin accessibility of neural crest cells throughout the course of development, the Crump lab utilized the zebrafish *Sox10-Cre* line to perform both single-cell RNA-seq and single-nucleotide ATAC-seq. These analyses provided clear evidence of distinct hyalin and elastic cartilage populations in the developing jaw and gills of zebrafish embryos, respectively. Single-cell RNA-seq and single-nucleotide ATAC-seq data further distinguished these populations by revealing mutually exclusive expression and chromatin accessibility of enhancers for isoforms of *upper zone of growth plate and cartilage matrix associated* (*ucma)* in either hyalin or elastic cartilage populations. Interestingly, comparative analyses of these two cartilaginous populations suggested that elastic cartilage in the gills represented the evolutionary equivalent of elastic cartilage in the outer ear and epiglottis of humans.

Next, Dr. Crump provided an example for how single-cell profiling allows for the identification of novel regulators of neural crest cell fate. Using SnapATAC, a software package for analyzing single-cell ATAC-seq datasets as a predictive model for gene expression, *nuclear receptor subfamily 5 group A member 2* (*nr5a2*) was identified as a gene exclusively expressed in the aboral side of the developing jaw. Conditional knockout experiments in both zebrafish and mouse embryos suggested that *nr5a2* prolonged multipotency in aboral domains to promote later tendon and glandular fates. Utilization of single-cell pipeline such as these could allow for more rapid and directed identification of regulators of neural crest cell fate.

Finally, by using a combination of single-cell technologies and retrogradely mapping the emergence of lineage-specific chromatin accessibility, Dr. Crump and colleagues identified a wealth of candidate lineage-priming factors for distinct populations of the zebrafish head, including *foxc1* in the cartilaginous lineage and *gata3* in the gill lineage. Together, these experiments suggested that rather than multilineage potential being an intrinsic property of neural crest cells, there was progressive and region-specific chromatin remodeling underlying acquisition of diverse lineage potentials throughout the craniofacial complex.

Dr. Pierre Le Pabic (University of North Carolina Wilmington) was the next speaker in the session and his presentation focused on defining regulators of endochondral growth that contributed to craniofacial bone size and shape. Previous studies in rodents determined that several cellular processes including cell enlargement (hypertrophy) and proliferation contributed to variation in size and shape of long bones; however, it remains unclear if these mechanisms are used to generate species variations within the craniofacial complex of

teleosts. Using two, closely related species of cichlid fishes with varying facial morphology and head length (*Copadichromis azureus* [CA] and *Dimidiochromis compressiceps* [DC]), Dr. Le Pabic and colleagues tested if growth zone activity was patterned in the embryo and what, if any, developmental loci regulated growth zone activity? To ask these questions, the Le Pabic lab focused on the development of the quadrate bone, which initially appears similar in size and shape in CA and DC embryos but ends up 26% larger in DC adults. To address the forces that contribute to this variable craniofacial morphology, the group first performed pulse-chase experiments. Calcein/Alizarin Red pulse chase experiments revealed a 62% increased growth rate in DC, suggesting that differential morphology between the species was a result of differential growth at the quadrate growth zone. Additional analyses examined possible cellular mechanisms that could account for or contribute to differential size of skeletal elements across species. Unlike what has been reported in rodents, cell enlargement through hypertrophy did not contribute to the overall size and shape differences between CA and DC species. Rather, cell proliferation appeared to play a more significant role with there being a 21% increase in the proliferating cell pool in DC embryos. Studies to determine the molecular mechanisms that drive these growth differences are ongoing as quantitative trait locus (QTL) analysis has identified a 1.5 MB region which includes interesting candidates. The future directions of the lab will include understanding the evolution of hypertrophy as a mechanism for species specific craniofacial variation.

Next, **Dr. Priyanka Kumari (University of Iowa)** presented her work on the use of massively parallel reporter assays (MPRAs) and genome engineering to identify candidates for noncoding variants that influence the risk for orofacial clefts. Most SNPs associated with orofacial clefting lie in noncoding DNA. To test the hypothesis that functional SNPs have allele-specific effects on enhancer activity and expression of neighboring risk-relevant genes, 8 genome-wide association study (GWAS)-identified loci containing 889 SNPs were used to conduct a MPRA in an oral epithelial cell line. MPRA results were filtered on genomic functional features for enhancer marks and validated by luciferase assays. Homology directed repair was used to engineer cells containing risk and nonrisk SNPs, while quantitative real time-polymerase chain reaction (qRT-PCR) and chromatin immunoprecipitation-quantiative PCR (ChIP-qPCR) were carried out to identify risk relevant genes and transcription factors, respectively. In vivo reporter assays were then carried out to validate functional SNPs and determine the etiology of orofacial clefts. This pipeline identified 66 of the 889 SNPs as having significant differences between risk and nonrisk allele activity. Interestingly, 46 of these 66 SNPs were located at the *IRF6* locus. Two SNPs within the *IRF6* locus (−9.7 and −21 kb) were particularly high scoring based on the presence of ATAC-seq and H3K27ac peaks, and most importantly were found to lie within human embryonic enhancer elements. Cells engineered with the −9.7 kb and −21 kb risk associated SNPs had significantly lower levels of *IRF6* expression. Based on this result, the group hypothesized these risk-associated SNPs impacted transcription factor binding. To test this hypothesis, the −21 kb SNP was further examined and determined to sit within the binding site for ETS1/2 and ERF transcription factors, thus providing a mechanism for reduced *IRF6* expression. Conditional analyses of both the 9.7 kb and −21 kb risk associated SNPs further supported their importance in orofacial clefts. Together, these data illustrated a method to identify noncoding variants that directly affect risk for orofacial clefts.

The final talk of the session was from **Claudio Macias-Trevino (Massachusetts General Hospital)**, who presented data detailing functional testing of human gene variants using a zebrafish model system. Alternative splicing is an important process that allows for epithelial to mesenchymal transitions of cell types essential for craniofacial development. *Epithelial splicing regulatory protein 1/2 (ESRP1/2)* represent genes that are both highly conserved and regulate tissue-specific alternative splicing. Although several human gene variants for ESRP1 and ESRP2 have been identified in patients with autosomal recessive deafness or cleft palate, the functional consequence of these variants was unclear. Mr. Macias-Trevino and colleagues applied an *esrp1/2* zebrafish mutant cleft model to interrogate the function of ESRP1 and ESRP2 human gene variants. Capped mRNAs harboring 20 ESRP1/2 human gene variants identified from cleft and other congenital cohorts were synthesized and individually injected into *esrp1/2*$^{-/-}$ mutant embryos and assayed for the ability to rescue the palate phenotype. A D222 frameshift mutation in ESRP1 resulted in loss of a functional protein product and failed rescue of the cleft phenotype. Four ESRP2 variants (R250Q, R315H, S508L, and R520stop) also failed to rescue the cleft phenotype present in *esrp1/2*$^{-/-}$ mutant embryos. Together these data represent a novel and innovative technique to utilize a zebrafish cleft model to functionally impute the pathogenicity of human gene variants when computational methods fall short.

4 | POSTER SESSIONS

Organizer: Dr. Amy Merrill, SCGDB Vice President (University of Southern California)

Each day ended with a 4-h "poster" session, in which faculty, postdocs, and students presented their poster abstracts in the form of a 5-min Zoom presentation. Winners of the poster presentation awards, sponsored by the American Association for Anatomy, were Michi "Stanley" Kanai (first), and a three-way tie for second place: Jung-Mi Lee, Soma Dash and Blanka Mrazkova in the postdoctoral fellow category, and Hung-Jhen (Olivia) Chen (first), Mathi Thiruppathy (second), and Chaofan Zhang (third) in the graduate student trainee category

Platform presentation awards sponsored by American Association for Dental, Oral, and Craniofacial Research (AADOCR). Winners of the inaugural platform presentation awards, which were sponsored by the AADOCR and recognized as impactful studies in craniofacial research, were Dr. Marco Trizzino in the junior faculty category, Dr. Casey Griffin in the postdoctoral fellow category, and Andrea Wilderman in the graduate student trainee category.

5 | SUMMARY

In summary, the 44th Annual Meeting of the Society for Craniofacial Genetics and Developmental Biology brought together a diverse group of scientists to present and discuss cutting edge approaches to expand our understanding of craniofacial development and alterations

that result in pathogenesis. The meeting continues to be an outstanding forum for the craniofacial sciences and a welcoming environment for all researchers regardless of career stage, gender or background. We look forward to reconvening for the 45th Annual Meeting of the SCGDB in person in 2022.

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All named authors/presenters have agreed to the publication of the information contained in this manuscript.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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