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Authors

Kline, Antonie D
Krantz, Ian D
Bando, Masashige
et al.

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Cornelia de Lange syndrome, related disorders, and the Cohesin complex: Abstracts from the 8th biennial scientific and educational symposium 2018

Antonie D. Kline[1], **Ian D. Krantz**[2,3], **Masashige Bando**[4], **Katsuhiko Shirahige**[4], **Stephenson Chea**[5], **Toyonori Sakata**[4], **Suhas Rao**[6], **Dale Dorsett**[7], **Vijay Pratap Singh**[8], **Jennifer L. Gerton**[8], **Julia A. Horsfield**[9], **Anne L. Calof**[5], **Olivia Katz**[2], **Marco Grados**[10], **Sarah Raible**[2], **Kristin Barañano**[11], **Gholson Lyon**[12], **Antonio Musio**[13], **Cheri S. Carrico**[14], **Douglas K. Clemens**[15], **Patti Caudill**[16], **Valentina Massa**[17], **Bryan E. McGill**[18], **Aila Dommestrup**[19], **Julia O'Connor**[19], **Richard E. Haaland**[20]

[1]Department of Pediatrics, Greater Baltimore Medical Center, Harvey Institute for Human Genetics, Baltimore, Maryland

[2]Division of Human Genetics, The Children's Hospital of Philadelphia

[3]Perelman School of Medicine at The University of Pennsylvania, Philadelphia, Pennsylvania

[4]Laboratory of Genome Structure and Function, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan

[5]Departments of Anatomy & Neurobiology, Developmental and Cell Biology, and the Center for Complex Biological Systems, University of California, Irvine, California

[6]Department of Structural Biology, Stanford University School of Medicine, Stanford, California

[7]Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, Saint Louis, Missouri

[8]Stowers Institute for Medical Research, and Department of Biochemistry and Molecular Biology, University of Kansas School of Medicine, Kansas City, Missouri

[9]Department of Pathology, Dunedin School of Medicine, The University of Otago, Dunedin, New Zealand

[10]Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland

[11]Child Neurology and Developmental Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland

[12]Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

[13]Institute for Genetic and Biologic Research, National Research Council, Pisa, Italy

Correspondence: Antonie D. Kline, Harvey Institute for Human Genetics, Greater Baltimore Medical Center, 6701 N. Charles St., Suite 2326, Baltimore, MD 21204., akline@gbmc.org.

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[14]Communication Sciences and Disorders, Elmhurst College, Elmhurst, Illinois

[15]Department of Oral Maxillofacial Surgery and Dentistry, Sinai Hospital of Baltimore, and Cross Keys Dental Associates, Baltimore, Maryland

[16]Milton J. Dance, Jr. Head & Neck Center, Greater Baltimore Medical Center, Baltimore, Maryland

[17]Department of Health Sciences, University of Milan, Milan, Italy

[18]Division of Pediatric and Developmental Neurology, Department of Neurology, Washington University, St. Louis, Missouri

[19]Kennedy Krieger Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland

[20]Research Department, Cornelia de Lange Syndrome Foundation, Avon, Connecticut

Abstract

Cornelia de Lange Syndrome (CdLS), due to mutations in genes of the cohesin protein complex, is described as a disorder of transcriptional regulation. Phenotypes in this expanding field include short stature, microcephaly, intellectual disability, variable facial features and organ involvement, resulting in overlapping presentations, including established syndromes and newly described conditions. Individuals with all forms of CdLS have multifaceted complications, including neurodevelopmental, feeding, craniofacial, and communication. Coping mechanisms and management of challenging behaviors in CdLS, disruption of normal behaviors, and how behavior molds the life of the individual within the family is now better understood. Some psychotropic medications are known to be effective for behavior. Other medications, for example, Indomethacin, are being investigated for effects on gene expression, fetal brain tissue, brain morphology and function in *Drosophila*, mice, and human fibroblasts containing CdLS-related mutations. Developmental studies have clarified the origin of cardiac defects and role of placenta in CdLS. Chromosome architecture and cohesin complex structure are elucidated, leading to a better understanding of regulatory aspects and controls. As examples, when mutations are present, the formation of loop domains by cohesin, facilitating enhancer-promotor interactions, can be eliminated, and embryologically, the nuclear structure of zygotes is disrupted. Several important genes are now known to interact with cohesin, including Brca2. The following abstracts are from the 8th Cornelia de Lange Syndrome Scientific and Educational Symposium, held in June 2018, Minneapolis, MN, before the CdLS Foundation National Meeting, AMA CME credits provided by GBMC, Baltimore, MD. All studies have been approved by an ethics committee.

Keywords

behavior; CdLS; cohesin complex; de Lange syndrome; loop domains; transcription regulation

Molecular update on the cohesinopathies and disorders of transcriptional regulation (DTRs) – A growing group of disorders with implications for understanding cornelia de lange syndrome (CdLS)

Kosuke Izumi[1,2], Sarah Raible[1], Maninder Kaur[1], Matthew Deardorff[1,2], Ian Krantz[1,2]

[1]Division of Human Genetics, The Children's Hospital of Philadelphia

[2]Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA

Germline and somatic mutations in structural and regulatory cohesin proteins including NIPBL, SMC1A, SMC3, RAD21, and HDAC8 are causative of CdLS. Cohesin, a multiprotein complex, plays a canonical role in regulating sister chromatid segregation during mitosis. A non-canonical role for cohesin in regulating gene expression is the likely mechanism underlying developmental disorders. Growing evidence supports a "transcriptome disruption model" for cohesinopathies and related diagnoses, including the implication of key transcriptional regulators (Super Elongation Complex, Mediator, Polycomb, CTCF, and others) with cohesin. A large number of genes/proteins are involved in the complexity of transcriptional regulation (from initiation, general transcription, elongation, pausing, backtracking, processing, termination, and associated epigenetic modification) and are increasingly being implicated in human developmental disorders when disrupted. The terms "transcriptomopathies" (coined by Yuan et al. [2015]), and more recently "Disorders of Transcriptional Regulation" or "DTRs" (coined by Izumi [2016]) have been proposed as a unifier of this group of molecularly linked diagnoses. An overview of these diagnoses will be presented.

The study for molecular function and dynamics of Nipbl in transcriptional regulation using a biochemical approach

Masashige Bando, Katsuhiko Shirahige

Laboratory of Genome Structure and Function, Institute for Quantitative Biosciences, The University of Tokyo, Tokyo, Japan

Mutations of *NIPBL* cause Cornelia de Lange syndrome, which exhibits intellectual deficiency and physical growth delay through a gene expressional regulation defect during development. Nipbl is known to basically function as a loader together with Mau2 to recruit cohesin onto chromosomes. Nipbl is a huge protein over 300 kDa and regulates transcription through the organization of chromosome structure by cohesin. However, the molecular mechanism for fulfilling the precise transcriptional regulation is not clear. To understand molecular dynamics and function, we analyzed this by a biochemical approach using an in vitro transcription assay.

Preinitiation complex (PIC) including RNA PolII, Mediators and GTFs in the nuclear extract (NE) were assembled on the template DNA dependent on GAL-VP16 as an activator in in vitro assay. Furthermore, we observed elongation factors and cohesin loader in the PIC.

Many factors containing RNA pol II were phosphorylated by CDK activated by the addition of dNTPs. During this time, the pausing factors, NELF, were accumulated with PIC in the presence of CDK9 inhibitor. We could detect the initiation, pausing, and elongation in each stage of transcriptional regulation. Cohesin loader is recruited at the same time when the PIC is formed occurring before the activation of RNAPII. To investigate the interacting partner with the cohesin loader we purified Nipbl/Mau2, and their interactions on the PIC assembled DNA were analyzed by Mass spectrometry. We found that mainly CDK8 modules of the Mediator complex were detected, and cohesin loader might be recruited by this module. Next, to examine how Nipbl affects PIC formation and RNAPII activation, we performed an in vitro assay using Nipbl or Mau2-depleted NE. Under this condition, PIC formation was not affected. However, the phosphorylation level of RNAPII was enhanced compared to mock depleted-NE. These results suggest that Nipbl might be required for the precise regulation of RNAPII activation.

Single-cell RNA sequencing studies to understand the origins of heart defects in a mouse model of Cornelia de Lange syndrome (CdLS)

Stephenson Chea, Henry Chang, Louise Villagomez, Martha Lopez-Burks, Arthur D. Lander, Anne L. Calof

University of California, Irvine, CA

Heart defects, including atrial septal defects (ASDs), ventricular septal defects, and outflow tract abnormalities, occur in about 30% in individuals with the most common form of CdLS, haploinsufficiency for *NIPBL*. In *Nipbl*± mice, heart defects occur at a similar frequency, but are primarily limited to ASDs. Recently, we investigated the contributions of different embryonic cell lineages to the development of ASDs by selectively creating or rescuing *Nipbl* haploinsufficiency in cardiac mesoderm, endoderm/endocardium, and neural crest (Santos et al., 2016). Creating *Nipbl* haploinsufficiency in either cardiac mesoderm or endoderm/endocardium produced the same cardiac phenotype as global *Nipbl* haploinsufficiency, whereas creating *Nipbl* haploinsufficiency in neural crest had no effect. Interestingly, in globally *Nipbl*± embryos, rescue of *Nipbl* haploinsufficiency solely in cardiac mesoderm or solely in endoderm/endocardium, were each able to rescue the ASD phenotype. These results imply that there is an additional, critical determinant of ASD risk that lies outside of what are considered to be the classic cardiogenic lineages. We hypothesize that this determinant reflects developmental coupling between body size and heart size, with defects arising when progenitor cells cannot be provided fast enough to meet the requirements imposed on the heart by other growing tissues. Since the *Nipbl*± embryo is small and thus does not require a normally sized heart, we propose that it can tolerate defective production of cardiac progenitors in one, but not multiple, cell lineages. In particular, we speculate that ASDs might arise as a result of a reduction in numbers or function of cardiac progenitor cells of the second heart field (SHF), which provide most of the cells that drive the early expansion of the heart, including cells that add to ventricles and atria, and form both the atrial septum and parts of the ventricular septum.

We therefore used single cell RNA sequencing (scRNA-seq) to investigate differences in the numbers/proportions of cardiac progenitors in *Nipbl*± and wildtype mice at embryonic day 7.5, a stage when cardiogenic progenitors are emerging from the primitive streak. We analyzed dissociated individual cells from four wildtype (*Nipbl Flox*/+) and four *Nipbl*± (*Nipbl FIN*/+) embryos (four total pools, two embryos of each genotype per pool) using droplet-based scRNA-seq (10X Genomics Chromium system; Illumina Hi-Seq). Bioinformatically-identified cells (>1,000 cells/pool) clustered into 18 presumptive cell types using their most differentially expressed genes as the primary criterion. Expression of known cell markers identified first heart field (FHF; *Mesp1-high*) and SHF (*Mesp1-low, Mef2c, Isl1*) cardiac progenitors, as well as more differentiated cardiac progenitors (*cTNT, Nkx2.5*). Observed differences in the proportions of progenitors suggested that *Nipbl*-haploinsufficient embryos are either markedly delayed or deficient in the generation of SHF, as compared with FHF, progenitors. Current studies are focused on confirming and quantifying these differences and understanding their consequences for heart development.

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Organization of 3D genome structure mediated by cohesin

Toyonori Sakata, Katsuhiko Shirahige

University of Tokyo, Tokyo, Japan

Genes coding cohesin and its accessory factors are frequently mutated in Cornelia de Lange syndrome (CdLS). Cohesin is essential in sister chromatid cohesion and also regulates transcription, contributing to insulation together with CCCTC binding factor (CTCF) and enhancer-promoter interactions. Because cohesin has a ring-like structure, chromatin loop models for transcriptional regulation have been proposed. It was also reported that cohesin and CTCF are enriched in topologically associating domain (TAD) boundaries, suggesting contribution to forming the structure. However, significance of cohesin and CTCF in organization of 3D genome structure remains unclear. To approach this question, we performed Hi-C using a human cell line, RPE, in which cells were depleted by RNAi either of a cohesin subunit, RAD21, a cohesin loader, NIPBL, or CTCF. We found that TADs largely disappeared both in the RAD21- and NIPBL-depleted cells. On the other hand, TADs remained and became larger in the CTCF-depleted cells. We also found that compartment structure was partially changed in the RAD21- and NIPBL-depleted cells and the changed structure was similar among those cells. In addition, we found that significant chromatin interactions were disrupted in the RAD21-depleted cells and the NIPBL-depleted cells, although CTCF-depletion showed a milder effect. Interestingly, we also found that enhancer-promoter interactions over TAD boundaries were newly emerged in these depleted cells. Taken together, our results suggest that cohesin is essential for formation of TADs and chromatin interactions and NIPBL and CTCF regulate proper formation of the structures.

Cohesin loss eliminates all loop domains

Suhas Rao[1,2,3], Su-Chen Huang[1,2], Brian Glenn St Hilaire[1,2,4], Jesse M. Engreitz[5], Elizabeth M. Perez[5], Kyong-Rim Kieffer-Kwon[6], Adrian L. Sanborn[1,4,7], Sarah E. Johnstone[5,8], Gavin D. Bascom[9], Ivan D. Bochkov[1,2], Xingfan Huang[1,10], Muhammad S. Shamim[1,2,10,11], Jaeweon Shin[1,10], Douglass Turner[1,12], Ziyi Ye[1,10], Arina D. Omer[1,2], James T. Robinson[1,5,12], Tamar Schlick[9,13,14], Bradley E. Bernstein[5,8], Rafael Casellas[6,15], Eric S. Lander[5,16,17], Erez Lieberman Aiden[1,2,4,5,10]

[1]The Center for Genome Architecture, Baylor College of Medicine, Houston, TX

[2]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX

[3]Department of Structural Biology, Stanford University School of Medicine, Stanford, CA

[4]Center for Theoretical Biological Physics, Rice University, Houston, TX

[5]Broad Institute of MIT and Harvard, Cambridge, MA

[6]Lymphocyte Nuclear Biology, NIAMS, NIH, Bethesda, MD

[7]Department of Computer Science, Stanford University, Stanford, CA

[8]Department of Pathology and Center for Cancer Research, Massachusetts General Hospital and Harvard Medical School, Boston, MA

[9]Department of Chemistry, New York University, New York, NY

[10]Departments of Computer Science and Computational and Applied Mathematics, Rice University, Houston, TX

[11]Medical Scientist Training Program, Baylor College of Medicine, Houston, TX

[12]Department of Medicine, University of California San Diego, La Jolla, CA

[13]Courant Institute of Mathematical Sciences, New York University, New York, NY

[14]NYU-ECNU Center for Computational Chemistry, NYU Shanghai, China

[15]Center of Cancer Research, NCI, NIH, Bethesda, MD

[16]Department of Biology, MIT, Cambridge, MA

[17]Department of Systems Biology, Harvard Medical School, Boston, MA

The human genome folds to create thousands of intervals, called “contact domains,” that exhibit enhanced contact frequency within themselves. “Loop domains” form because of tethering between two loci—almost always bound by CTCF and cohesin—lying on the same chromosome. “Compartment domains” form when genomic intervals with similar histone marks cosegregate. Here, we explore the effects of degrading cohesin. All loop domains are

eliminated, but neither compartment domains nor histone marks are affected. Loss of loop domains does not lead to widespread ectopic gene activation, but does affect a significant minority of active genes. In particular, cohesin loss causes superenhancers to colocalize, forming hundreds of links within and across chromosomes, and affecting the regulation of nearby genes. We then restore cohesin and monitor the reformation of each loop. Although reformation rates vary greatly, many megabase-sized loops recovered in under an hour, consistent with a model where loop extrusion is rapid.

Brca2 controls cohesin function and regulates the same genes as cohesin

Ziva Misulovin, Michelle Pherson, Maria Gause, Dale Dorsett

Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, Saint Louis, Missouri, USA

The cohesin complex mediates sister chromatid cohesion to ensure that daughter cells receive the correct number of chromosomes upon cell division. Cohesin also helps regulate genes that are crucial for growth and development. Minor deficits in proteins that regulate cohesin, such as NIPBL, or HDAC8 cause Cornelia de Lange syndrome (CdLS). CdLS is also caused by mild missense mutations in cohesin subunits. The potential roles of the Pds5 cohesin regulator, and the Wapl and Brca2 proteins that interact with Pds5, in CdLS are unknown. We investigated how Pds5, Wapl, and Brca2 influence cohesin function in Drosophila. Pds5 and Wapl work together, likely during DNA replication, to control how far cohesin spreads out along chromosomes, thereby determining which active genes bind cohesin. Pds5 is required for sister chromatid cohesion and Brca2 opposes this Pds5 function. However, Pds5 and Brca2 work together to facilitate control of gene expression by cohesin, and homozygous *Brca2* mutations reduce growth. Brca2 is essential for homologous DNA repair, and our studies expand the known roles for Brca2 by showing that it also regulates sister chromatid cohesion and gene expression. *BRCA2* mutations in humans increase the frequency breast and other cancers, and our findings raise the possibility that *BRCA2* mutations may also influence the severity of CdLS.

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The role of placenta in the etiology of Cornelia de Lange syndrome

Vijay Pratap Singh, Jennifer L. Gerton

Stowers Institute for Medical Research, Kansas City, MO

CdLS is a rare human developmental syndrome caused by mutations in genes that support the function of the cohesin complex. Much work has been done to evaluate and characterize embryonic development in mouse models for CdLS (Haberland, et al. 2009, Kawauchi, et al. 2009). CdLS mutations can compromise several aspects of embryonic development including bone, limb, heart, and gut formation. One very common feature associated with CdLS is slow growth and small size. Mouse embryos with CdLS mutations are considerably

smaller in utero and after birth and survive at sub-Mendelian ratios. Early work indicated that PAPP-A, a protein produced by the placenta, was often low during pregnancy with a CdLS conceptus (Arbuzova, et al. 2003, Westergaard, et al. 1983). As placenta size often scales with embryo size, one possible explanation for this observation is that the smaller embryos have smaller placenta that are deficient in producing PAPP-A. However, a second possibility is that the placenta itself is dysfunctional due to the CdLS mutation, independent of the embryo. Recent work revealed the high prevalence of placentation defects and their contribution to abnormal embryo development in mice (Perez-Garcia, et al. 2018). We examined whether placental dysfunction might contribute to embryonic developmental phenotypes associated with mouse models of CdLS. We find that placental size and development are negatively affected by loss of *Hdac8* and *Nipbl*, independent of the effect on embryonic development. Gene expression analysis from *Nipbl* or *Hdac8* placenta showed activation of a common inflammatory pathway. Knockout of protein kinase R (PKR) (Yang, et al. 1995), which activates downstream targets of the pathway, partially rescues embryo survival and bone formation in *Nipbl* and *Hdac8* mutant embryos. Our data suggest placental defects significantly contribute to the development of CdLS phenotypes in mouse models.

A zebrafish model for cohesin function in early embryo development

Michael Meier[1], Jenny Grant[1], Amy E. Dowdle[1], Amarni Thomas[1], Jennifer Gerton[2], Philippe Collas[3], Justin M. O'Sullivan[4,5], Julia A. Horsfield[1,5]

[1]Department of Pathology, University of Otago, Dunedin 9016, New Zealand

[2]Stowers Institute for Medical Research, 1000 E 50th Street, Kansas City, MO, USA

[3]Department of Molecular Medicine, Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo, Norway

[4]Liggins Institute, The University of Auckland, Private Bag 92019, Auckland, New Zealand

[5]Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Private Bag 92019, Auckland, New Zealand

During the first few hours of existence, early zygotic cellular events are regulated by maternally inherited molecules. From a defined time point, the zygotic genome gradually becomes active and is transcribed. At zygotic genome activation (ZGA), changes in chromatin structure are associated with new transcription immediately following the maternal-to-zygotic transition (MZT).

The nuclear architectural protein, cohesin, and CCCTC-binding factor (CTCF), contribute to chromatin structure and gene regulation in a variety of cell types. We found that normal cohesin function is important for genome activation in zebrafish. Partial depletion of cohesin subunit Rad21 delays ZGA without affecting cell cycle progression. In contrast, CTCF depletion has little effect on ZGA whereas complete abrogation is lethal. Genome-wide profiling of Rad21 binding reveals a change in distribution from pericentromeric satellite

DNA sequences and few locations including the *miR-430* locus (whose products are responsible for maternal transcript degradation), to gene regulatory elements as embryos progress through the MZT. After MZT, a subset of Rad21 binding overlaps pioneer factor Pou5f3, which activates early expressed genes. Rad21 depletion disrupts the formation of nucleoli and RNA polymerase II foci, suggestive of global defects in chromosome architecture.

We propose that Rad21/cohesin redistribution to active areas of the genome is key to the establishment of chromosome organization and the embryonic developmental program. Our findings suggested that development is affected from the very start of embryogenesis in the cohesinopathies; human syndromes characterized by compromised cohesin function.

Bicornuate uterus in Cornelia de Lange syndrome: Overlap with Hand-Foot-Genital syndrome and HOXA involvement

Antonie Kline[1], Matthew Deardorff[2], Amy Kimball[1], Natalie Blagowidow[1]

[1]Harvey Institute for Human Genetics, Greater Baltimore Medical Center, Baltimore, MD

[2]Division of Genetics, Children's Hospital of Philadelphia, Department of Pediatrics, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA

Female genital tract malformations are rare, occurring in 0.4–4.3% of the fertile female general population (Grimbizis et al., 2001; Byrne et al., 2000), and higher if infertility and sex chromosome abnormalities are included. Defects occur in the Mullerian system, initiated and influenced by many genetic factors during embryogenesis, when there is failure of or incomplete fusion of the two Mullerian ducts. Although genital malformations including cryptorchidism, micropenis and hypospadias are noted in over half of males with Cornelia de Lange syndrome (CdLS), a disorder of the cohesin protein complex, few genital deformities have been reported in females.

As part of our long-term adolescent and adult multidisciplinary aging clinic, a pelvic and abdominal ultrasound is performed on all patients if tolerated. In addition to assessing for renal malformations, the female genital tract is evaluated. Following ultrasounds of 24 females age 12 to 54 years, notably six (25%) were found to have a bicornuate uterus. Most ovaries were normal in appearance, otherwise no other uterus pathology was noted, and no ambiguous or intersex genitalia were seen (initial findings reported in Kline et al., 2015).

Few genetic syndromes present with Mullerian fusion anomalies such as bicornuate uteri, which typically do not cause medical complications, other than pregnancy difficulties. Bicornuate uteri have been seen in a few syndromes, including Roberts syndrome (Freeman et al., 1974), another disorder of the cohesin protein complex. The Hand-Foot-Genital syndrome (HFGS) includes genital malformations in both males and females, including Mullerian fusion defects. Specific extremity findings include small hands and feet, short great toes, and other minor anomalies. Several reported patients have had additional findings such as: a female with speech delay (Pezzani et al., 2015); a male with feeding problems, poor weight gain, gastroesophageal reflux, obstructive sleep apnea, peripheral

pulmonic stenosis, diaphragmatic hernia, and scoliosis (Tas et al., 2016); and a male with mild dysmorphic features such as prominent philtrum and small chin, as well as global developmental delay (Yokoyama et al., 2017).

Many of the HFGS findings can also be seen in CdLS. Half of the females in our cohort with CdLS and bicornuate uteri have short great toes, and half have brachyclinodactyly of the fifth fingers. HFGS is due to mutations or deletions in *HOXA13* in the *HOXA* gene cluster on 7p15. Cohesin is known to coordinate *HOXA* chromatin structure and gene expression (Wang et al., 2015) and maintain associated domains at *HOXA* (Nwigwe et al., 2015) during development. We hypothesize that the chromatin-coordinating role of cohesin is related to proper expression of the *HOXA* cluster. If this activity is disrupted, this could lead to uterine fusion anomalies and limb features, as well as other features overlapping with those seen in CdLS.

Towards a rational approach to identify potential targets for pharmacological therapies for Cornelia de Lange syndrome

Anne Calof[1], Martha E. Lopez-Burks[1], Louise Villagomez[1], Henry Chang[1], Adam Stabell[1], Felix Grun[1], Shimako Kawauchi[1], Rosaysela Santos[1], Dale Dorsett[2], and Arthur Lander[1]

[1]University of California, Irvine, CA

[2]St. Louis University. School of Med., St. Louis, MO

Cornelia de Lange syndrome (CdLS) is most commonly caused by haploinsufficiency for *Nipbl*, a gene that encodes a cohesin-associated protein that has global effects on transcription. *Nipbl* deficiency causes hundreds of subtle gene expression changes in every tissue, and animal model studies indicate that synergistic and/or combinatorial actions of these transcriptional changes likely cause the deficits observed in CdLS. We are using mouse models of CdLS, and cells derived from these mice, to test whether drugs that increase *Nipbl* expression can correct downstream pathological gene expression changes, and potentially restore function. Here, we studied indomethacin (Indo), a drug we and others have shown to increase *Nipbl* expression by as much as twofold in cultured cells and cell lines, and which can ameliorate CdLS-like phenotypes in *Drosophila* (Dorsett et al., 2016; Kline et al., 2017 and unpublished observations).

In initial tests, we focused on the nervous system, as cognitive and behavioral deficits are particularly pronounced in CdLS, and much of brain development occurs postnatally. We generated a conditional-invertible *Nipbl* allelic series that permits us to carry the *Nipbl* mutation on inbred mice, simplifying molecular and genetic analysis (Santos et al., 2016). We used this allelic series to make mice haploinsufficient for *Nipbl* in the nervous system (Nestin-Cre;Nipbl$^{Flox/+}$ mice), and used RNA sequencing to develop a gene expression signature (GES) for *Nipbl*-deficient cortex. Q-RT-PCR was used to evaluate expression of 15 GES genes in cortical tissue of mutant and control mice fed for 4 weeks on control chow or chow containing Indo at the highest doses that were sufficiently well-tolerated by the animals. Treatment failed to significantly alter expression of any GES genes, including

Nipbl itself, in either mutant or wildtype cortex (Calof et al., 2017; Kline et al, 2017). Mass spectrometry indicated that Indo levels in brains of treated mice were only 10–30 nM, 4–5 orders of magnitude less than levels required to achieve a reliable *Nipbl* mRNA increase in cultured mouse embryonic fibroblasts (MEFs). Multiple experiments in which pregnant dams were treated with Indo also failed to detect a significant increase in *Nipbl* expression in treated fetal tissues, although fetal tissue levels were 10- –30-fold higher than in adult brain.

These results, coupled with a significant incidence of adverse health effects in mice treated with these Indo doses, led us to conclude that Indo treatment is not likely to be a viable therapy for CdLS, if the goal is to increase *Nipbl* expression in treated individuals. Nonetheless, analysis of gene expression changes effected by Indo in model systems or cells lines could still provide insight into targets for drug therapies. Interestingly, effects of Indo on cultured cell lines and on *Drosophila* are unlikely to be related to Indo's known role as a COX inhibitor, since *Drosophila* has only one Cox gene and that is expressed only in the female germline. To identify relevant target(s) of Indo, we are performing RNA sequencing to identify all genes significantly affected by Indo in MEFs, and are comparing these with genes whose expression is affected by treatment with another COX inhibiting drug that has no effect on *Nipbl* expression in MEFs. Initial results are promising, showing a robust response in gene expression changes with Indo treatment in both wildtype and mutant MEFs. Results of these studies will be discussed.

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Investigating Indomethacin and Acemetacin efficacy in normalizing expression levels of known Cornelia de Lange syndrome dysregulated genes in human-derived fibroblasts

Chiara Bettale, Maninder Kaur, Sarah Raible, Marissa Benedict, Jason A. Mills, Dale Dorsett, Ian D. Krantz

Division of Human Genetics, The Children's Hospital of Philadelphia and the Perelman School of Medicine at the University of Pennsylvania

Individuals with CdLS present with significant cognitive and behavioral issues including verbal, social, emotional deficits as well as autism spectrum disorder. Sleep abnormalities, repetitive, and self-injurious behaviors are also characteristic in some individuals with CdLS. Psychotropic medications are currently used to ameliorate behavioral complications in children with CdLS, but response to treatments are very individualized and few studies have reported on the clinical management of these behaviors. Results from our clinical survey will be used to quantify the overall prevalence of medication use in this patient population and evaluate the effectiveness of current treatments for CdLS. Previous studies in individuals with CdLS and *NIPBL* mutations have identified conserved patterns of significantly dysregulated genes (Liu et al., 2009), suggesting a role for *NIPBL* in regulating several key developmental and neural pathways. However, further evidence is needed to establish *NIPBL* as a CdLS therapeutic target. Indomethacin and its derivative acemetacin have recently been identified as drugs able to normalize *NIPBL* expression levels (Dorsett,

2016). In this study, we tested the efficacy of indomethacin and acemetacin to normalize expression patterns in CdLS fibroblast cell lines. We evaluated the expression levels of 10 genes (*NIPBL, FGD6, PAPSS2, TRERF1, LTB, RHOBTB3, ARHGAP24, PHF16, ROBO1, ZNF608*) that were previously shown to be significantly dysregulated in *NIPBL* individuals (2009). *MYC*, a known target of *NIPBL* activity that is significantly downregulated in *NIPBL* mutant cell lines, was also included. This study aims to analyze the clinical use and overall effectiveness of current treatments for CdLS, provide further evidence for indomethacin and acemetacin as potential CdLS medications, and further demonstrate *NIPBL*'s potential as a CdLS therapeutic target. Results of these studies will be presented.

A drug rescue study of neurological deficits in a Drosophila model of Cornelia de Lange syndrome

Olivia L. Katz[1], Ian D. Krantz[1,2], Yaning Wu[1]

[1]Division of Human Genetics, The Children's Hospital of Philadelphia

[2]Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania

Individuals with Cornelia de Lange syndrome (CdLS) display diverse developmental deficits, including growth, limb, and multiple organ abnormalities, and intellectual disabilities. Severely affected individuals most often have dominant loss-of-function mutations in the *Nipped-B-Like* (*NIPBL*) gene, and milder cases often have missense or in-frame deletion mutations in genes encoding components of the cohesin complex. Previous studies have shown that modest decreases in *NIPBL* and cohesin activity alter the transcription of many genes that regulate growth and development. The cohesin complex is highly conserved across species, allowing us to study *Drosophila* models of CdLS.

As nearly all patients with CdLS have intellectual disability as well as other neurologic and/or psychiatric differences, we have been particularly interested in investigating neurologic and behavioral phenotypes in our fly models. Much of our work has been with flies that are heterozygous for a loss-of-function mutation in the *Nipped-B* gene, the fly ortholog of human *NIPBL*. We have found that *Nipped-B* heterozygous mutant Drosophila exhibit learning and memory deficits, sleep disturbances, circadian arrhythmicities, morphological brain abnormalities, and global gene expression changes. These phenotypes align closely with the neurological and behavioral differences seen in CdLS patients, confirming that heterozygous Drosophila *Nipped-B* mutants provide a useful model for understanding CdLS.

Indomethacin is a nonsteroidal anti-inflammatory agent used in a variety of contexts including to treat arthritic swelling and pain, improve certain forms of kidney dysfunction, and in newborns, to close patent ductus arteriosis (PDAs). Recently, indomethacin was identified in a drug screen as a compound that is able to reverse *Nipped-B* dependent wing and eye defects in *Drosophila* mutants. It has also been shown to increase *Nipped-B* mRNA levels in cultured *Drosophila* cells and increase *NIPBL* mRNA levels in CdLS patient fibroblasts and cultured embryonic fibroblasts from *Nipbl*(±) mice. The ability

of indomethacin to improve brain development and neurologic outcome had not been previously assessed.

We have shown that indomethacin treatment, starting from the larval stage of *Drosophila* development, significantly improves the brain morphology defects and learning and memory deficits of *Nipped-B* mutant heterozygotes, suggesting it may hold promise for bettering neurodevelopmental prognoses for patients with CdLS. As the classic form of the diagnosis is caused by haploinsufficiency of *NIPBL*, we hypothesize that indomethacin may improve aspects of CdLS pathogenesis by boosting expression from the functional *NIPBL* allele, allowing it to compensate better for the loss of function of the other allele. Accordingly, we are testing this hypothesis with quantitative PCR techniques.

A double-blind cross-over study of N-Acetylcysteine in Cornelia de Lange syndrome for repetitive and self-injurious behaviors

Anupriya Razdan[1], Nirmal Singh[2], Priyadurga Kodi[1], Marco Grados[1]

[1]Johns Hopkins University School of Medicine, Baltimore, MD

[2]All India Institute of Medical Science, New Delhi, India

Objective:

To conduct a double-blind cross-over study of N-Acetylcysteine (NAC) for repetitive and self-injurious behaviors (RB-SIB) in 10 children and adults with Cornelia de Lange syndrome (CdLS).

Background:

RB-SIB occur in up to one-half of individuals with CdLS, with SIB constituting a particularly malignant subset of RBs, for which treatment options are often limited. Using paradigms useful in the treatment of OCD and related disorders, this pilot trial will test the efficacy of NAC in reducing RB-SIB in CdLS. NAC acts by decreasing reactive oxygen species, a robust antioxidative stress and neuroprotective mechanism. In CdLS, neuronal oxidative stress may occur due to a deficit of the cohesin component rad21, a deficit which can lead to dysregulation of the expression of the transcription factor runx1 (Horsfield, 2007). Runx1, in turn, is key in the neuronal developmental system that supports the proliferation of GABAergic interneurons (Stifani 2008). GABA interneurons are critical in maintaining the GABA-glutamate (inhibitory-excitatory) neurotransmitter homeostasis in brain neurocircuitry; thus, it is plausible that a CNS hyperglutamatergic/high oxidative stress state may characterize CdLS. NAC has been shown to decrease a form of SIB, skin picking, in normotypical adults and is a safe drug with only mild GI distress as a common side effect.

Methods:

A double-blind cross-over design consisting of 18 weeks, with phases: (1) 8 week pill/placebo; (2) 2 week washout; (c) 8 week placebo/pill. The aim is to recruit 10 participants who have clinically valid suprathreshold scores on measures of repetitive behaviors/SIB:

Aberrant-Behavior Checklist (ABC) stereotypies and irritability score (SIB) as well as compulsivity scores on the Yale-Brown Obsessive–Compulsive Scale for PDD (CY-BOCS-PDD). NAC dosing will start 600 mg daily, increased weekly by 600 mg as tolerated, up to 1800 mg daily.

Result:

Primary efficacy measures are that the Aberrant Behavior Checklist stereotypies and irritability (SIB) score show a decrease of >35%. Secondary measures include adaptive functioning (Vineland Adaptive Behavior Scales), change in caregiver burden. All outcomes are measured at baseline 1, week 8, baseline 2 and week 8 (crossover).

Conclusion:

Pilot efficacy data on use of NAC to decrease RB-SIB in CdLS can provide an effect size to plan for future clinical trials in CdLS.

Prospective attitudes toward treatment among parents of children with Cornelia de Lange syndrome

Antonie D. Kline[1], Joanne Kimball[2], Bonnie Royster[2], Sarah Raible[3], Ian Krantz[3], Richard Haaland[2]

[1]Harvey Institute for Human Genetics, Greater Baltimore Medical Center, Baltimore, MD

[2]Cornelia de Lange Syndrome Foundation, Avon, CT

[3]Division of Human Genetics, The Children’s Hospital of Philadelphia, Philadelphia, PA

Treatment of nonmetabolic genetic conditions has been a controversial topic ethically, legally, and from the family/patient point of view. For a number of single gene disorders, there is precedent for therapeutic compounds demonstrating positive results for specific measurement tools. For Cornelia de Lange syndrome (CdLS), there have been many publications about management of clinical findings and recommendations for early intervention therapy and development of skills, but no specific treatment medication protocols to date.

A focus group was formed in 2012 to promote biennial discussions about future treatment for CdLS among clinical, allied health, basic science, and lay members of the CdLS Foundation, the national parents support organization. Because of the established pattern of identifying potential treatments in various animal models, then moving to a trial on human subjects with more widespread investigations, the group wanted to be prospectively prepared before presenting such a trial to parents of children with CdLS. Thus, a voluntary anonymous questionnaire for parents/caregivers was devised and distributed. Questions included prioritization of various medical and developmental aspects of CdLS, opinions on participating in studies which might improve quality of life, and open-ended questions on what would be important as a family and reasons for or against participation.

Approximately 1,500 parents/caregivers received the survey, and there were 203 respondents (14%). The majority of parents/caregivers who completed the surveys stated that they would consider enrolling their child in a study that might improve the quality of life of their child, specifically related to feeding, early intervention and using an already approved drug. There was equal interest and noninterest in a study using a non-FDA approved drug, 40% for each. Questions about location of study, need for travel, time involved, coordination with child's living situation or other medications, and/or specific goals of treatment were raised, all helpful for our future planning. The surveys showed that there is good reception for future studies, particularly for improvement of the quality of life of the child with CdLS, and predominantly related to the GI system, communication and behavior. Further details of the surveys will be discussed.

Somatic mosaicism in Cornelia de Lange syndrome (CdLS)

Sarah E. Raible[1], Maninder Kaur[1], Jim Zhang[1], Ian D. Krantz[1,2]

[1]Division of Human Genetics, The Children's Hospital of Philadelphia

[2]Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA

Cornelia de Lange Syndrome is a multisystem developmental diagnosis with variable growth, cognitive, craniofacial, limb, gastrointestinal, cardiac, and other systemic involvement. A diagnosis of CdLS can be confirmed by molecular testing of the five known CdLS genes (including *NIPBL, SMC1A, SMC3, HDAC8,* and *RAD21*). Approximately 65% of individuals with a clinical diagnosis are found to have a positive test result, when testing blood, for one of the five known CdLS genes. This leaves about 35% of individuals without a defined molecular etiology and a confirmed diagnosis. With such a large percentage of individuals without genetic explanation or confirmation of a diagnosis, we suspect there are additional mechanisms involved in CdLS yet to be discovered.

Most recently, somatic mosaicism for *NIPBL* has been reported in a small percentage of individuals (Huisman et al. 2013). Single case reports of somatic mosaicism have also been reported in *SMC1A* and *SMC3* (Ansari et al. 2014). Mosaicism occurs when an individual contains two or more cell lines that are genetically distinct. Mosaic mutations are most often not detected through standard screening approaches using DNA isolated from blood. Given these findings, complete testing would require examining a different tissue type in addition to blood to rule out a mosaic mutation in other tissues. At the Center for Cornelia de Lange Syndrome and Related Diagnoses at Children's Hospital of Philadelphia (CHOP) we are interested in further examining the prevalence of mosaicism in CdLS as this mechanism may explain a large portion of the ~35% of individuals that have negative testing for the five known CdLS genes in their blood. Molecular confirmation is critical for establishing a diagnosis, ending a diagnostic odyssey for a family, understanding long-term prognostication, accurately identifying recurrence risk, and delivering proper care and ongoing medical management. Demonstrating that a child is mosaic as the cause of their diagnosis would indicate that the parents are not at risk for germline mosaicism as a mosaic child would have arisen as a postzygotic event.

To date, we have identified mutations in DNA isolated from lymphocytes in over 300 probands by CSGE/Sanger sequencing/MLPA/SNP array. Mutations including missense, nonsense, small deletions and insertions, splice site mutations, and genomic rearrangements have been identified in these genes. In 30 to 35% of our cohort A causative mutation has not been identified. This could be due to a number of possibilities including: (1) technical limitations (mutations are in regions of these genes not detected by currently used screening methodologies); (2) mosaicism with levels in blood (most commonly used tissue source for DNA diagnostics) too low to detect or absent; (3) causative mutations lie in other genes yet to be discovered. Prior studies have primarily focused on screening for *NIPBL* mosaicism; however, there are several additional genes implicated in CdLS that can present in a mosaic form. We have conducted preliminary mosaicism screening on a cohort of 25 individuals with a clinical diagnosis of CdLS. Two had a mosaic *NIPBL* mutation in DNA isolated from buccal sample identified on NGS and sanger confirmed. We are interested in further investigating the frequency of *NIPBL* mosaicism in addition to exploring prevalence of mosaicism in the five additional CdLS genes as well as several other genes that present with an atypical CdLS phenotype.

Phenotypic evaluation of patients with truncating SMC1A mutations and intractable epilepsy

Kristin W. Barañano[1], Amy Kimball[2], Susan Fong[1], Alena S. Egense[3], Catherine Hudon[4], Antonie D. Kline[2]

[1]Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD

[2]Harvey Institute for Human Genetics, Greater Baltimore Medical Center, Baltimore, MD

[3]Division of Human Genetics, Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD

[4]Department of Human Genetics, McGill University, Montreal, Quebec, Canada

Cornelia de Lange syndrome (CdLS) is a rare developmental malformation syndrome characterized by small stature, limb anomalies, distinctive facial features, developmental delays, and behavioral issues. The diagnosis of CdLS is typically made clinically or by an identified mutation in one of the genes associated with CdLS. SMC1A mutations are the cause of approximately 5% of the cases of CdLS. SMC1A is located on the X chromosome and is thought to escape X-inactivation in some, but not all, females. Patients with SMC1A mutations are being increasingly identified on the basis of next generation sequencing technologies, without necessarily a clinical suspicion of CdLS prior to the test result. In general, intractable epilepsy is not considered a prominent feature of CdLS, yet this is a feature of many of these patients newly diagnosed with mostly truncating SMC1A mutations.

We report on a series of patients with mostly truncating *SMC1A* mutations and intractable epilepsy. Eligible patients were recruited based on communication with their treating clinicians, through outreach from the Cornelia de Lange Foundation, and through an

SMC1A Facebook group. Patients were clinically evaluated through the multidisciplinary aging clinic for Cornelia de Lange syndrome held twice a year at Greater Baltimore Medical Center, where patients receive education about CdLS and are evaluated by providers in a number of disciplines. Their concerns are discussed and recommendations are synthesized. As part of this study, patients with *SMC1A* mutations underwent the same structured genetic and neurological examinations and a standardized interview regarding their epilepsy. In addition, medical records were reviewed.

In contrast with patients with typical *SMC1A*-associated CdLS, all of the identified patients were female, and when available, X-inactivation studies were usually highly skewed. In general, a clinical diagnosis of CdLS had not been entertained prior to the genetic testing results. Here, we describe the physical appearance of the participants and compare this to the criteria for classical CdLS. We also report on the clinical characteristics of their epilepsy, including age of onset, types of seizures, EEG findings and response to various antiepileptic medications. These findings allow us to draw conclusions about how this population of patients with mostly truncating *SMC1A* mutations fit into the spectrum of CdLS and the broader spectrum of cohesinopathies and allow generalizations that may impact clinical care and, in particular, epilepsy management.

Expanding the phenotypic and genetic characterization of TAF1 syndrome

Sara Ballouz[1], Max Dörfel[1], Jonathan Crain[1], Megan Crow[1], Laurence Faivre[2,3], Catherine E. Keegan[4], Sophia Kitsiou-Tzeli[5], Maria Tzetis[1], Jesse Gillis[1], Gholson J. Lyon[1]

[1]The Stanley Institute for Cognitive Genomics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA

[2]GAD team, INSERM UMR 1231, Université Bourgogne Franche-Comté, Dijon, France

[3]Centre de Référence Maladies Rares Anomalies du Développement et FHU TRANSLAD, CHU Dijon, Dijon, France

[4]Department of Pediatrics, Division of Genetics and Department of Human Genetics, University of Michigan, Ann Arbor, MI, USA

[5]Department of Medical Genetics, National Kapodistrian University of Athens, Athens, Greece

We previously described the discovery of a new X-linked genetic syndrome, with severe intellectual disability (ID), a characteristic caudal prominence, and distinctive facial features, associated with missense variants in the X-chromosomal gene TAF1, encoding the largest subunit of the general transcription factor IID (TFIID) multiprotein complex. Recent work toward understanding the molecular basis of Cornelia de Lange syndrome (CdLS) has implicated mutations in TAF6, a component of TFIID, as playing an important role in the pathogenesis of this syndrome, drawing a link between the molecular etiology of TAF1 syndrome and CdLS. CdLS is a phenotypically and genetically heterogeneous syndrome characterized by distinct facial features, hirsutism, developmental delay, intellectual

disability, and limb abnormalities. Thus, a proportion of CdLS-like cases may be due to molecular causes that are similar to those of TAF1 syndrome—namely, a disruption of normal TFIID function. The phenotypic spectrum of CdLS includes phenotypes that are shared by TAF1 syndrome affected individuals, such as anteverted nares, long philtrum, high palate, low-set ears, hearing impairment, intellectual disability, microcephaly, and strabismus. We have now found an additional 21 families with missense variants in TAF1, which has allowed us to expand the phenotypic spectrum of TAF1 syndrome and also has enabled phenotypic comparisons to individuals with CdLS. We also performed RNA sequencing from RNA isolated directly from blood from six of the previously identified TAF1 syndrome families, and we found an "outlier gene," CACNA1I, that recurs in five of six pedigrees with the TAF1 syndrome.

Gene expression dysregulation in Cornelia de Lange syndrome (CdLS) and CdLS-like cells

Dubravka Cukrov, Patrizia Sarogni, Antonio Musio

Institute for Genetic and Biomedical Research, National Research Council, Pisa, Italy

Cornelia de Lange syndrome (CdLS) is a rare multisystem disease characterized by prenatal and postnatal growth delay, craniofacial abnormalities, intellectual impairment, limb anomalies, hypertrichosis, and defects in the cardiopulmonary and gastrointestinal systems. CdLS is genetically heterogeneous and is caused by mutations in the *NIPBL, SMC1A, SMC3, RAD21,* and *HDAC8* genes belonging to the cohesin pathway. Although cohesin was first identified as playing a critical role in holding sister chromatids together, experimental evidence indicates that cohesin also plays a role in regulating gene expression. This is substantiated by the findings that cellular and developmental models of CdLS display modest perturbations in gene expression. Recent experimental data indicate that mild forms of CdLS overlap phenotypically with other diseases such as Rubinstein-Taybi syndrome (RTS) and KBG syndrome, with which it shares the typical growth retardation, intellectual disability and facial features, making the correct diagnosis of CdLS very difficult. During our research, we plan to identify specific pathways by RNA-seq that can explain the overlapping phenotypes among CdLS, RTS and KBG.

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Feeding and Related Issues in Individuals with Cornelia de Lange Syndrome

Cheri S. Carrico

Communication sciences and disorders, Elmhurst college

Individuals affected with Cornelia de Lange syndrome (CdLS) exhibit atypical facial, physical, and developmental characteristics associated with challenges in oral feeding and speech and language development. Physical characteristics, such as micrognathia, small

dentition, a weak bite, and a cleft or highly arched palate, can impact oral feeding. Gastroesophageal reflux, tube feeding, autistic tendencies, and sensory issues are linked with food aversions. Delayed physical growth, gastroesophageal reflux disease, and possible failure to thrive may be associated with poor nutritional intake.

Speech-language difficulties also are prevalent among individuals with CdLS. Although there is no known link between feeding difficulties and communication deficits in other populations, prior evaluation of 45 children ages 1–13 years with CdLS noted no or minimal oral language, with markedly delayed acquisition of speech.

Because feeding issues are prevalent among individuals with CdLS, an assessment of feeding has been ongoing since 2004 based on direct observation of feeding and questionnaires completed by caregivers. Areas of investigation include observed feeding behaviors, difficulties associated with feeding, caregiver concerns and associated medical entities. Observations on 130 affected individuals, age 3 months through 36 years, have been collected to date. New findings include an increased incidence of choking, coughing, gagging, vomiting, and spitting food out at meal times; refusal to feed orally; and oral defensiveness. Additional reported health concerns include gastroesophageal reflux disease. The recommendations generated from this work will be discussed.

Lower mandibular labial frenum in Cornelia de Lange syndrome

Douglas K. Clemens[1,2], Antonie D. Kline[3], Bethany Serafin-Awalt[1,4]

[1]Department of Oral Maxillofacial Surgery and Dentistry, Sinai Hospital of Baltimore, Baltimore, MD

[2]Cross Keys Dental Associates, Baltimore, MD

[3]Harvey Institute for Human Genetics, Greater Baltimore Medical Center, Baltimore, MD

[4]Valley Village Oral Surgery Associates, Owings Mills, MD

A number of dental and oral complications and anomalies have been reported in Cornelia de Lange syndrome (CdLS). These include: delayed primary tooth eruption, delayed loss of primary teeth, missing teeth, submucous, or overt cleft palate, and loss of enamel secondary to gastroesophageal reflux disease. Dental evaluation of individuals with Cornelia de Lange syndrome should occur twice annually when younger, and three times annually in adulthood, and ongoing home care is an important part of good oral hygiene in CdLS.

Labial frenula are malformations of the oral vestibule, and surgical division has been recommended when clinically significant (Mueller and Callanan, 2007). These have not been associated with plaque formation or gingivitis (Addy et al., 1987), but gingival inflammation should be controlled (Powell and McEniery, 1982) and surgery can be postponed.

A recent finding of a prominent mandibular labial frenum has been noted in patients with CdLS. It is not associated with gap between the two lower central incisors, as can be seen

commonly from a maxillary labial frenum in the upper incisors. The unusual lower frenum causes retraction and can lead to eventual severe anterior recession of the gingiva around the lower incisors, with periodontal disease and eventual bone loss. We have evaluated numerous patients with CdLS over the years, and have recently noted this finding in at least five patients during early adolescence each having gingival recession on permanent teeth #24 and #25. Frenectomies have been completed on two patients by our group to date under sedation for dental rehabilitation. Electrocautery was used to incise through the lower labial thick frenum down to the level of the periosteum. The periosteum was removed from the underlying bone to release tension on the gingiva, with excellent results.

These findings have prompted a new recommendation in CdLS. To correct the recession, these patients should undergo a release of the labial mandibular frenum by having a frenectomy performed by an oral surgeon or periodontist. Several cases and this procedure will be discussed.

Expanding communication options in individuals with CdLS using high technology Augmentative Alternative Communication

Amanda Matrone[1], Patti Caudill[2]

[1]Towson University, Towson, MD

[2]Milton J Dance, Jr Head & Neck Center, Greater Baltimore Medical Center, Baltimore, MD

Cornelia de Lange syndrome (CdLS) is a genetic disorder associated with cognitive communication deficits. These deficits can range from mild to severe and are impacted by comorbid diagnoses including: social anxiety, selective mutism, intellectual disability, autism, cleft palate, hearing deficits, and motor speech disorders. Augmentative Alternative Communication (AAC) is a term applied to a variety of aided and nonaided, low technology and high technology nonspeech communication methods. Informal observations during CdLS clinics suggest Augmentative Alternative Communication is underused in individuals with CdLS. In addition, parent/caregiver interviews reveal limited understanding of AAC devices, where to obtain an AAC evaluation, and funding availability for devices.

An overview of high tech AAC will be presented, including: low technology vs high technology AAC, high technology AAC options, precursor behaviors suggesting an individual is a candidate for use of a high technology AAC device, where to obtain AAC evaluations, current laws and reimbursement issues related to high technology AAC, funding options for high technology AAC and barriers to high technology AAC use (Ganz et al., 2017; Meder and Wegner, 2015). Results of a pilot study on the use of AAC by individuals with CdLS will be presented.

Cornelia de Lange syndrome: Neurodevelopmental impairment in experimental models

Valentina Massa

Department of Health Sciences, University of Milan, Italy

Cornelia de Lange Syndrome (CdLS) can result from mutations in at least five genes: *NIPBL, SMC1A, SMC3, RAD21,* and *HDAC8*, all coding for components of the cohesin complex. The cohesin complex regulates the structure and organization of chromosomes, repair damaged DNA and gene expression regulation. We have previously exploited *D. rerio* (zebrafish) models of *nipbl*, *smc1a* and, more recently, *hdac8* obtained by morpholino antisense injections for studying embryonic development in cohesin loss-of-function. In cohesin-zebrafish embryos, hindbrain (i.e., the embryonic precursor of cerebellum, medulla, and pons) development was impaired and increased cell death was observed specifically located in abnormal structures. Moreover, canonical WNT-pathway, a known master regulator of central nervous system pattering was found down-regulated. This finding was corroborated by the effects of chemical activation of canonical WNT pathway in loss-of-function embryos, which resulted in rescuing of adverse phenotypes and restoring physiological levels of cell death. Interestingly, studying protein levels in human primary fibroblasts, canonical WNT pathway down-regulation was observed also in *NIPBL*- and *SMC1A*-mutated patient-specific fibroblasts compared to donors. In order to deepen the analysis of neural development in models of CdLS, murine CdLS Neural Stem Cells (NSCs) have been used. Wild-type NSCs were tested by down-regulating HDAC8 activity using a specific inhibitor, coupled with siRNA strategies (for silencing hdac8) in both proliferating and differentiating cells. In all tested NSCs with inhibited HDAC8 activity, reduced proliferation, increased cell death and decreased differentiation toward the neuronal lineage was observed.

Abnormal microglia and enhanced inflammation-related gene transcription in mice with conditional deletion of Ctcf in Camk2a-Cre-expressing neurons

Bryan E. McGill,[1] Ruteja A. Barve,[2] Susan E. Maloney,[3] Amy Strickland,[4] Nicholas Rensing,[1] Peter Wang,[4] Michael Wong,[1] Richard Head,[2] David F. Wozniak,[3] Jeffrey Milbrandt[4]

[1]Division of Pediatric and Developmental Neurology, Department of Neurology

[2]Genome Technology Access Center, Department of Genetics

[3]Department of Psychiatry

[4]Department of Genetics, Washington University, St. Louis, MO

CCCTC-binding factor (CTCF) is an 11-zinc finger DNA-binding domain protein that regulates gene expression by interacting with cohesin and modifying 3D chromatin structure. Human mutations in CTCF cause intellectual disability and autistic features reminiscent of Cornelia de Lange syndrome. Knocking out Ctcf in mouse embryonic neurons is lethal by neonatal age, but the effects of CTCF deficiency in postnatal neurons are less well studied. We bred *Ctcf*^{loxP} mice (Heath et. al, 2008) with *Camk2a-Cre* mice (Tsien et. al, 1996) to knock out *Ctcf* postnatally in glutamatergic forebrain neurons. *Ctcf*$^{loxP/loxP}$; *Camk2a-Cre* (*Ctcf* CKO) mice of both sexes were viable and exhibited

profound deficits in spatial learning/memory, impaired motor coordination, and decreased sociability by 4 months of age. *Ctcf* CKO mice also had reduced dendritic spine density in the hippocampus and cerebral cortex. Microarray analysis of mRNA from *Ctcf* CKO mouse hippocampus identified increased transcription of inflammation-related genes linked to microglia. Separate microarray analysis of mRNA isolated specifically from Ctcf CKO mouse hippocampal neurons by ribosomal affinity purification identified upregulation of chemokine signaling genes, suggesting crosstalk between neurons and microglia in *Ctcf* CKO hippocampus. Finally, we found that microglia in *Ctcf* CKO mouse hippocampus had abnormal morphology by Sholl analysis and increased immunostaining for CD68, a marker of microglial activation. Our findings confirm that *Ctcf* KO in postnatal neurons causes a neurobehavioral phenotype in mice and provide novel evidence that CTCF depletion leads to overexpression of inflammation-related genes and microglial dysfunction.

Behavioral assessment and treatment of problem behavior in children with Cornelia de Lange syndrome

Aila Dommestrup[1], Michelle A. Frank-Crawford[1], Julia T. O'Connor[1,2], Patricia F. Kurtz[1,2], Michelle D. Chin[1], Louis Hagopian[1,2]

[1]Kennedy Krieger Institute

[2]Johns Hopkins University School of Medicine, Baltimore, MD

The presence of problem behavior such as self-injury and aggression is a prominent and challenging feature of Cornelia de Lange syndrome (CdLS). Despite the growing body of research on behavior problems associated with behavioral phenotypes in genetic disorders, problem behavior in this population is not well understood. Additionally, few studies have examined environmental influences on problem behavior in CdLS (Arron et al., 2006; Moss et al., 2005). Recently, researchers have sought to identify "functional behavioral phenotypes" of problem behavior that include: types of problem behavior, variables maintaining problem behavior, stimulus preferences, and responsiveness to treatment (Frank-Crawford et al., 2016).

Preliminary findings from functional analyses and other assessments in CdLS indicate differences in forms of problem behavior, functions for problem behavior, and responsiveness to certain classes of stimuli, relative to other genetic disorders. The results from a comparison of individuals with CdLS to individuals with Down (DS) and fragile X syndrome (FXS) will be presented. Preliminary data from eight individuals with CdLS, ages 8 to 21 years, who received behavioral services for severe problem behavior will be discussed. With regard to form of problem behavior, individuals with either CdLS or FXS were more likely to exhibit self-injury relative to those with DS. Additionally, preference assessment results indicated that auditory stimuli were most preferred by CdLS participants, relative to those with FXS (visual, nonedible). For individuals with CdLS, functional analysis results indicated that access to adult attention and escape from work may play a role, as indicated among five of eight participants. Function-based interventions to reduce problem behavior were prescribed and in all cases resulted in a reduction of 80% or

greater relative to baseline. These data suggest that problem behavior in CdLS is associated with environmental factors and is responsive to behavioral intervention. Taken together, our preliminary results suggest that some classes of stimuli may impact preferences and function of problem behavior; and further examination of such variables may lead to more precise and targeted interventions.

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