7th International RASopathies Symposium: Pathways to Understanding – Expanding Knowledge, Enhancing Research and Therapeutic Discovery

July 23-25, 2021
VIRTUAL MEETING

Chairs
Maria Kontaridis, PhD, Masonic Medical Research Institute
Amy Roberts, MD, Boston Children's Hospital

Honorary Chairs: Marco Tartaglia, PhD, Bambino Gesù Children's Hospital, and *Martin Zenker, MD, Universitätsklinikum Magdeburg*

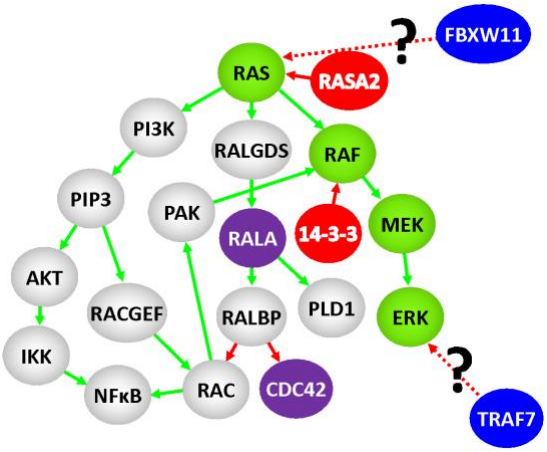
SESSION 1: Genes, Pathways and Genocopies

Moderator: Katherine A. Rauen MD PhD, UC Davis, CA

CDC42, RASA2, FBXW11, ~~ZNF426~~, YWHAZ, TRAF7: Novel RASopathy genes or not?	Martin Zenker MD, Institute of Human Genetics, University Hospital Magdeburg, Germany

In this contribution, recently reported new associations of genes / gene variants with RASopathies or RASopathy-like phenotypes are reviewed. Contrary to the title in the agenda, ZNF426 was skipped, for which no explicit assertion has been made to be a RASopathy-associated gene, but instead RALA was included, which has been reported in 2019 as causing a RASopathy-like disorder. Assessment of the genes' associations to RASopathies considered the number of observations, the quality of phenotypic overlap, the evidence of causality for the variant, the link to the RAS-MAPK pathway, and the experimental evidence for its dysregulation by the mutant proteins.

For all six genes, the number of cases reported to have a RASopathy / RASopathy-like disorder is currently too small to establish robust case-level evidence for them to be novel RASopathy genes. For FBXW11and TRAF7, the RASopathy-like phenotype has only been observed in some of the mutation carriers; for CDC42, one specific mutation has been proposed to be associated with a Noonan-like phenotype, while others are not. Experimental evidence for mutations in those genes having impact on the RAS-MAPK signaling pathway varies considerably.



Overall, these new proposed candidate genes can be grouped in three categories (as shown in the figure):

RASA2 and YWHAZ remain the strongest candidates through their direct link as negative regulators of RAS and RAF, but the evidence on the case level as well as on their precise impact on the RAS-MAPK pathway needs further confirmation.

CDC42 and RALA are components of a non-canonical RAS effector pathway. These pathways may contribute to some of the features of individuals with "classical" RASopathies and thereby explain some overlap with RASopathies in some CDC42 / RALA mutation carriers, but neither functional nor case level evidence are sufficient to call them RASopathy genes.

FBXW11 and TRAF7 have an uncertain link to the RAS-MAPK pathway and the observed Noonan-like features in a small fraction of mutation carriers may rather be a phenocopy than the correlate of a critical impact on RAS-MAPK signaling.

Novel disease gene identification for RASopathies: where are we now?

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Molecular genetics of RASopathies. Common themes and novel mechanisms in RAS signaling dysregulation.

RAS proteins are small monomeric GTPases that function as molecular switches controlling a major intracellular signaling network that, depending on the cellular context, guides diverse biological functions such as proliferation, migration, survival, cell fate determination, differentiation, and senescence. Within this network, signal flow through the RAF-MEK-ERK protein kinase pathway, the first described mitogen-associated protein kinase (MAPK) cascade, controls early and late developmental processes, including determination of morphology, organogenesis, synaptic plasticity, and growth. Signaling through the RAS-MAPK cascade is tightly controlled, and its enhanced activation has been known for decades to represent a major event in oncogenesis. Activating somatic *RAS* gene mutations occur in approximately 30% of human cancers, but the upregulation of this signaling pathway can also result from enhanced function of upstream signal transducers or RAS effectors, as well as from inefficient function of feedback mechanisms.

Unexpectedly, discoveries derived from a massive disease gene hunting effort performed in the last 17 years have established a novel scenario in which the upregulation of this signaling cascade underlies a group of clinically related developmental disorders, the RASopathies, sharing facial dysmorphism, cardiac defects, reduced postnatal growth, ectodermal and skeletal anomalies, variable cognitive deficits, and susceptibility to certain malignancies as major features. These disorders are caused by heterozygosity for mutations in genes encoding RAS proteins, regulators of RAS function, modulators of RAS interaction with effectors, or downstream signal transducers. The majority of these disease genes have been identified by using a hypothesis-driven approach based on "gene candidacy", and the collection of data on the molecular spectrum of mutations in each of these genes has allowed to appreciate the differential impact of RASopathy-causing and cancer-associated mutations on protein function and intracellular signaling. Remarkably, these discoveries have also permitted to identify novel molecular mechanisms driving dysregulated RAS signaling able to perturb certain developmental processes but that apparently do not contribute significantly to oncogenesis, as well as to recognize "common themes" in the dysregulation of RAS signaling underlying the RASopathies.

More recently, the "hypothesis-free" strategy based on the sequencing of the exome, the protein-coding portion of the genome, in trios with sporadic Noonan syndrome, the most common and clinically variable among RASopathies, has allowed to identify disease genes that are more remotely related to the RAS-MAPK signaling backbone, expanding the concept of "RASopathy gene". Consistent with the data collected by dissecting the extent and branching of signaling dysregulation driven by RASopathy-causing mutations, these new findings further document the relevance of additional signaling pathways in disease pathogenesis in the RASopathies. Due to the differential perturbing impact elicited by individual mutations on intracellular signaling, and the high level of cross-talk with other signaling pathways, a more accurate and systematic characterization of the functional consequences of RASopathy mutations is required for the development of therapeutic interventions to efficiently restore proper intracellular signaling and ameliorate the major postnatal issues altered in RASopathies.

Noncanonical GTPases: RRAS2, RRAS, MRAS, RIT1

Tetsuya Niihori, Koki Nagai, Taiki Abe, Shin-Ichi Inoue and **Yoko Aoki**
Department of Medical Genetics, Tohoku University School of Medicine, Sendai, Japan

The Ras superfamily of small GTPases comprises a group of molecular switches that regulate diverse cellular functions. Among the Ras superfamily members, Ras subfamily members play a predominant role in cell proliferation. There are more than 30 GTPases in the RAS subfamily. In addition to the classical RAS, RRAS, RRAS2, MRAS, and RIT1 are included. Like classical RAS, GTPase-deficient variants of non-canonical GTPases can cause the growth transformation of NIH3T3 cells. RRAS, RRAS2, MRAS, and RIT1 share 48%, 55%, 57%, and 44% sequence identity with HRAS, respectively. These GTPases have an N- or C-terminal extension. The sequence identity of the effector domain is very high, but hypervariable regions are unique and are predicted to have different lipid modifications.

In 2013, *RIT1* mutations were identified in patients with Noonan syndrome using whole-exome sequencing. *RIT1* germline mutations were clustered in the G1, G2 and Switch II regions. Two mouse model with *Rit1* mutations have been generated. Both mutant mice showed craniofacial changes and the increased heart/weight volume. Subsequently, germline mutations in *RRAS*, *MRAS* and *RRAS2* have been identified in patients with Noonan syndrome.

In this talk, the structures, somatic or germline mutations, clinical characteristics, and functional analysis of non canonical GTPases was presented.

RASopathy genetic variation in biobanks	Bruce Gelb MD, Mount Sinai, NYC, NY

Purpose: The purpose of this study is to use a genotype-first approach to explore the RASopathies using biobank data.
Methods: This study uses exome sequencing and corresponding phenotypic data from Mount Sinai's BioMe (n = 32,344) and the United Kingdom Biobank (UKBB; n = 200,000). Variant curation identified pathogenic/likely pathogenic (P/LP) variants in RASopathy genes that were rated definitive in ClinGen.

Results: Eighty-seven subjects harbored P/LP RASopathy variants; four (4.6%) were diagnosed, one met van der Burgt diagnostic criteria for Noonan syndrome (NS) and another 52% had >1 classic NS feature. Mean height Z scores for BioMe and UKBB were -0.9 and -0.6, respectively (both significant results). However, heights less the 3rd percentile and the 10th percentile were noted in 10 and 23 individuals, respectively. Major and minor NS features such as hypertrophic cardiomyopathy, pectus deformity and ptosis were observed in modest numbers of individuals. Bleeding issues were noted in 13 of the 87 persons harboring a RASopathy variant. The prevalence of hypothyroidism/autoimmune disorders (14/87 persons) was enriched compared to the biobank populations (p = 0.001).

Conclusions: Rates of persons harboring a RASopathy variant in BioMe and UKBB were 1:2300 and 1:2700, respectively. Few were diagnosed with a RASopathy, but a substantial fractions of individuals harboring P/LP variants exhibited partial or full phenotypic matches to these traits. Overall, there was a reduced rate of RASopathy cardinal features compared to expectations. Autoimmune disorders, particularly hypothyroidism, was increased. Routine screening of thyroid functions tests in adults with NS might be indicated.

SESSION 2: Novel Strategies and Mechanisms in RASopathies

Moderators: Pau Castel PhD, NYU Langone; Annette Bakker PhD, Children's Tumor Foundation, NYC, NY

Intronic CRISPR repair in a preclinical model of LZTR1-associated cardiomyopathy	Lukas Cyganek PhD, University Med Center, Göttingen, Germany

Our understanding of the pathophysiological alterations and mechanisms in Noonan syndrome remains limited and effective therapeutic options are lacking. By generating induced pluripotent stem cell–derived cardiomyocytes from two affected siblings with biallelic variants in LZTR1, we were able to recapitulate the hypertrophic phenotype in vitro and uncovered a causal link between LZTR1 dysfunction, RAS–MAPK signaling hyperactivity, hypertrophic gene response and cellular hypertrophy. In a proof-of-concept approach, we explored a clinically translatable intronic CRISPR repair and demonstrated a rescue of the hypertrophic phenotype.

Using iPSCs to study syndrome-specific properties	L. Legler[1]; Y. Sun[1], D. Bohler[1], Ross Cagan[2]; B. Gelb[3]; M.I. Kontaridis[1]

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RAF1 (or c-RAF) plays a central role in cell proliferation, differentiation and apoptosis. Pathogenic variants in RAF1 have been implicated in RASopathies, where children present with clinical features consistent with Noonan syndrome (NS) and >95% have hypertrophic cardiomyopathy (HCM). Because Raf1 knock-in RASopathy mice have an HCM phenotype that can be prevented by MEK inhibition, we sought to determine if rigosertib, a novel dual MEK and PI3K inhibitor in phase III clinical trials for treatment of chronic myelomonocytic leukemia, could reverse HCM in Raf1L613V/+ mice. To do this, we first measured the cardiac functional parameters in vehicle or 3-week drug-treated (100 mg/kg body weight i.p. twice daily) C57/Bl6 (WT) or Raf1L613V/+ mice (4 males and 4 females for each genotype and treatment condition). Echocardiography revealed that, as compared to vehicle-treated females, Raf1L613V/+ treated female mouse hearts had normalized chamber dimensions and posterior wall thicknesses. In addition, total heart mass of Raf1L613V/+ treated females, as well as the size of their individual cardiomyocytes, normalized to levels similar to those of WT. In contrast, Raf1L613V/+ treated male mice were less responsive to the 3-week treatment with rigosertib; while posterior wall thicknesses decreased, the overall left ventricular mass remained similar to Raf1L613V/+ vehicle-treated male hearts, suggesting that perhaps the 3-week time period for the study was insufficient to completely reverse the HCM in the male mice. Despite this, overall, the data strongly indicate that rigosertib may have potential clinical benefit in reversing RAF1-associated HCM. Moreover, in addition

to the cardiac phenotype, we interestingly also observed reversals of other RASopathy-associated pathologies in response to rigosertib treatment. Specifically, normalization of tibia length, indicative of enhanced bone growth, and of ocular hypertelorism were both observed in the rigosertib-treated animals. In summary, rigosertib normalized and reversed RASopathy-associated HCM, as well as other syndromic features, in Raf1L613V/+ mouse hearts. These data indicate that rigosertib, which was identified from a large-scale fly screen and found to be effective against most fly RASopathy lines, could be considered a potential first-in-use treatment for RASopathies.

How to temper Shoc2-ERK1/2 signals -molecular insights	Emilia Galperin Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY 40536, USA

Signaling scaffolds guide the flow of information and the spatial organization of the enzymes within the ERK1/2 signaling pathway. However, the mechanisms that control assembly and dynamics within scaffolding complexes, as well as the mechanisms regulating the cellular distribution of these complexes, remain largely unknown. We unravel a novel, multi-level paradigm in which allosteric modifications alter the ability of the scaffold protein Shoc2 to actively accelerate ERK1/2 signals. Shoc2 facilitates the ERK1/2 pathway by tethering to proximity essential enzymes of the pathway, Ras and RAF-1. Germ-line mutations in Shoc2 disrupting the spatial distribution of Shoc2 or its ability to assemble the signaling complex lead to a congenital disorder with a wide spectrum of developmental abnormalities, Noonan-like syndrome with loose anagen hair.

Our lab studies mechanisms that fine-tune ERK1/2 signals transmitted through the Shoc2 complex. We found that Shoc2 assembles an elegant multi-component complex that incorporates several proteins of the ubiquitin system. To fine-tune amplitude of ERK1/2 signal transmitted via the complex, Shoc2 tethers the E3 ligase HUWE1, the (AAA+) ATPases, PSMC5 and VCP/p97 and the deubiquitinating enzyme, USP7. All of these enzymes are integral to the intricate feedback mechanism by which ubiquitination controls the amplitude of the Shoc2-ERK1/2 signals.

Our studies show that while HUWE1 ubiquitinates Shoc2 and RAF-1, ATPases modulate the ubiquitination of Shoc2 and RAF-1 through the remodeling of the complex. We demonstrated that PSMC5 and VCP/p97 are involved in the recruitment of the Shoc2 complex to endosomes where it undergoes remodeling. Importantly, our recent studies show that, in the context of the Shoc2 complex, USP7 functions as a "molecular switch". In the Shoc2 complex USP7 "activates" HUWE1 thereby triggering the mechanisms that "tunes-off" the ERK1/2 signals transmitted. Congenital Shoc2 mutations affecting Shoc2 interaction with USP7 lead to aberrant Shoc2 ubiquitination and signal transmission.

In summary, our studies demonstrate that the Shoc2 scaffold employs multi-protein enzymatic machinery to govern the amplitude of Shoc2-ERK1/2 signals. We also uncover novel molecular mechanisms underlying the pathogenesis of Noonan-like syndrome with loose anagen hair. Overall, these studies significantly advance our understanding of the mechanisms by which non-enzymatic scaffolds regulate specificity and dynamics of the ERK1/2 signaling networks.

Novel findings and expansion of phenotype in a mosaic RASopathy caused by somatic KRAS variants	Caitlin Chang MD, FCCMG, FRCPC, British Columbia Women & Children's Hospital, Vancouver BC, Canada

Mosaic KRAS variants and other RASopathy genes cause oculoectodermal, encephalo-cranio-cutaneous lipomatosis, and Schimmelpenning-Feuerstein-Mims syndromes, and a spectrum of vascular malformations, overgrowth and other associated anomalies, the latter of which are only recently being characterized. We present a cohort of 8 patients with mosaic KRAS variants and their associated features. We expand the association with embryonal tumors, including the third report of embryonal rhabdomyosarcoma, as well as novel findings of Wilms tumor and nephroblastomatosis in two individuals. Rare or novel findings in our series include the presence of epilepsy, polycystic kidneys, and T-cell deficiency in one individual, and multifocal lytic bone lesions in two individuals. Finally, we describe the first use of targeted therapy with a MEK inhibitor for an individual with a mosaic KRAS variant. Given the potential increased risk of cancer, we suggest consideration for cancer surveillance with periodic ultrasound of the abdomen in childhood.

SESSION 3: Lymphatic and Cardiovascular Manifestations
Moderators: Sahar Mansour MD, St Georges Hospital, London, UK; David Stevenson MD, Stanford Univ CA

Lymphatic problems and lymphedema in RASopathies	Kristiana Gordon MD, St. Georges Hosp, London, UK

The lymphatic system is responsible for fluid homeostasis and immune surveillance within the body. Primary lymphoedema occurs as a result of a developmental abnormality of the lymphatic system due to a genetic fault. RASopathies are associated with primary lymphoedema and lymphatic anomalies. These include hydrops, lower limb and genital lymphoedema and central conducting lymphatic anomalies. We present a series of patients with Noonan syndrome and complex lymphatic anomalies to highlight the challenging problems they face. We also present a case of using oral Trametinib to successfully manage progressive lymphatic failure in a young man with Noonan syndrome.

Patterns and therapeutic options in Noonan syndrome-related lymphedema	Maxim Itkin MD, Penn Medicine, Philadelphia, PA

Patients with Noonan syndrome often present with clinical signs of the lymphatic disorders. Recent development of lymphatic imaging techniques, such as dynamic contrast enhanced MR lymphangiography and Intranodal Lymphangiography allow for new insight into the anatomy and pathology of the lymphatic system. One of the main findings in almost all patients with Noonan syndrome is the increase of the lymphatic flow rate. This can be explained by either the increase in the permeability of the capillaries or a venous insufficiency and/or venous obstruction.

The increase in the lymphatic flow rate results in lymphangiectasia, that in turns causes lymphatic valve insufficiency and lymphatic reflux. The clinical presentations of lymphatic reflux include chylothorax, chylous ascites, protein losing enteropathy and genital lymphorrhea. Novel treatment approaches include interstitial/mesenteric lymphatic embolization, percutaneous and surgical thoracic duct decompression, all which allow successful treatment of these conditions.

All patients with clinical symptoms of lymphatic disorders must undergo lymphatic imaging and possible treatment. Systematic imaging study of the lymphatic system in Noonan would help to better understand the effects of the changes in the lymphatic system on patients' health.

Therapeutic strategies for the treatment of hypertrophic cardiomyopathy in Noonan syndrome with multiple lentigines	Anton Bennett PhD, Yale School of Medicine, New Haven, CT

Noonan syndrome with multiple lentigines (NSML) is an autosomal dominant disorder presenting with hypertrophic cardiomyopathy (HCM). Up to 85% of NSML cases are caused by mutations in the PTPN11 gene that encodes for the Src homology 2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP2). Prof Bennett and his group have utilized a 'knock in' mouse model of NSML – ptpn11Y279C/+ which develops HCM by week 12 of life. They have found that a transmembrane Shp2 binding protein called protein zero-related (PZR) is increased in its level of phosphorylation and binding in the hearts of NSML mice. When a mutation of PZR that blocks Shp2 binding is introduced into NSML mice HCM is blocked and this correlated with inhibition of AKT – suggesting that the interaction between PZR and Shp2 drives NSML-associated HCM. They used an inhibitor called Dasatanib (Sprycel) which already has FDA approval for chronic myeloid leukemia in adults and acute lymphoblastic anemia in children to block the binding between PZR and Shp2. Professor Bennett described multiple experiments using a dose of Dasatanib that was up to 100-fold lower than that used to treat cancer patients in the mouse model of NSML. He demonstrated low toxicity and low lethality. He showed that Dasatanib inhibited PZR tyrosyl phosphorylation in the heart of these mice even at very low doses. He did echocardiograms on the mice and proved that low dose Dasatinib, used before 12 weeks prevented the development of HCM in the mouse and prevented fibrosis of the heart muscle. He also gave the drug at 14 weeks after the HCM presented and demonstrated reversal of the hypertrophy in the cardiac muscle. In conclusion, this is a potential therapeutic agent for HCM in patients with NSML.

KEYNOTE & POSTER SESSION
Moderator: Beth Stronach PhD, RASopathies Network, Pittsburgh PA

Opening Keynote: **Update on MEK and mTOR inhibition for RASopathy-associated cardiac disease**	Gregor Andelfinger MD PhD, CHU Ste-Justine, Montreal, Canada, 2019 Penn Medicine MDBR Grant Recipient

RASopathies are a syndromic spectrum of disorders affecting multiple organs and caused by germline mutations in the RAS/MAPK pathway. They can cause progressive RASopathy-associated cardiomyopathy (RAS-CM), for which no preventive or curative therapies

exist. Specifically, infants presenting with heart failure within the first months of life suffer from high morbidity and mortality. If presenting later in life, RAS-CM contributes to morbidity, and there is an increased risk for sudden death. Treatment options at this point are purely symptomatic, such as heart failure therapy, respiratory support, extracorporeal circulation, catheter or surgical intervention, and cardiac transplantation. However, animal studies and limited case reports have shown beneficial effects on cardiac disease of small molecule therapies targeting specifically activated pathways in certain RASopathies, such as mTOR or MEK-inhibition.

Here, we report on 25 patients from Europe and North America with molecularly verified RASopathies and progressive myocardial hypertrophy and/or life-threatening symptoms in whom we initiated off-label or compassionate inhibition of the mTOR or MEK pathways after exhaustion of standard therapies. Over a follow-up period of 295.5 patient-months (median, 5.5 months; range, 1.5-50), we observe a decrease of mortality in critically ill patients less than 6 months of age treated with mMEKi and/or mTORi as compared to standard care and described in natural history observational studies (25% vs. 60.9%, p=0.031, Fisher´s exact test). Avoidance of surgical intervention occurred in 64.7% (11 of 17 patients in which surgical outflow tract resection was indicated), and clinical meaningful improvement (greater than 20% from baseline) of equal or greater than 50% of cardiac outcome parameters assessed in 18 of 25 patients (72%) of patients undergoing MEKi and/or mTORi treatment.

This data suggests that selected RASopathy patients may benefit from mechanism-informed therapeutics guided by the biological understanding of cardiac pathology in this disease spectrum.

SESSION 4: Neurocognitive Associations

Moderators: Tamar Green MD, Stanford University, A; Marni Axelrad PhD, Texas Children's, Houston, TX

Non-NF1 RASopathies, Particularly Noonan syndrome, are associated with multi-focal low-grade gliomas which commonly harbor FGFR1 mutations

Alberto Broniscer MD MS, University of Pittsburgh Medical Center (UPMC) Children's Hospital of Pittsburgh

Preliminary results of a multi-institutional study addressing the association of CNS cancers with non-NF1 RASopathies will be shared. The majority of subjects had Noonan syndrome and were younger than 18 years. The most commonly found cancers were low-grade gliomas, which usually had an indolent behavior. Based on the study design, no conclusions can be taken about the incidence of CNS cancers in this population. No systematic screening for CNS cancers is currently recommended for children with Noonan syndrome.

Neurological and neurodevelopmental features of CFC syndrome: a multinational study

Rene Pierpont PhD, University of Minnesota, Minneapolis, MN

Purpose: Neurological complications are common in RASopathies and are especially challenging in the management of cardiofaciocutaneous (CFC) syndrome. The current study evaluated whether variability in neurological phenotype, including the frequency and severity of seizures, adaptive functioning, and gross motor functioning, was associated with results from molecular genetic testing of individuals with CFC.

Methods: A multinational cohort of caregivers of individuals with CFC was recruited. Neurological and neurodevelopmental information pertaining to 138 individuals with molecular confirmation of CFC syndrome (BRAF=90; MAP2K1=36; MAP2K2=10; KRAS=2) was obtained from review of medical records and a caregiver-completed electronic survey. Severity of seizures among study participants was measured using an adapted version of the Early Childhood Epilepsy Severity Scale (E-Chess). Caregivers were administered validated measures of adaptive and gross motor function.

Results: History of seizures was nearly twice as common among individuals with BRAF or MAP2K1 variants (57-61%) as compared to MAP2K2 variants (30%), and seizures tended to be more severe in these subgroups as well. Seizures were not present in either of the children with KRAS variants. Specific molecular variants were identified that caused a disproportionate incidence of severe seizures, including infantile spasms. Adaptive and gross motor skills varied widely across individuals with CFC, and were associated both with the presence and severity of seizures as well as the genotype of study participants.

Conclusion: Molecular genetic testing results can aid in the prediction of epilepsy in CFC syndrome. Successful management of seizures in CFC is associated with key neurodevelopmental outcomes. Neurological phenotypes represent an important target for future clinical trials.

Epilepsy and BRAF mutations: phenotypes, natural history, and genotype-phenotype correlations	Domenica Battaglia MD, Fondazione Policlinico Universitario A Gemelli IRCCS, Catholic University, Rome, Italy

Cardiofaciocutaneous syndrome (CFCS) is a rare developmental disorder caused by upregulated intracellular signaling through the RAS-MAPK pathway. In most patients, signaling dysregulation is due to de novo activating missense mutations in BRAF, encoding a serine/threonine protein kinase belonging to the backbone of the MAPK cascade. Neurological involvement is a common feature of the disorder, with moderate to severe developmental delay/intellectual disability representing an invariant feature. Children with CFCS are also prone to epilepsy, which is a major life-threatening complication of the disorder. To date in literature a systematic assessment of the electroclinical features, natural history of disease, long-term outcome and response to therapy is lacking.

We report on an observational longitudinal study performed on 34 patients with molecularly confirmed diagnosis (11 males, mean age of 15.8 years ± 10.6, mean follow-up of 9.2 years ± 4.7). For all patients we performed neurological examination, cognitive assessment when possible, neuroimaging, electrophysiological assessment, systematic assessment of epilepsy features. Correlation analyses were performed taking into account gender, age at seizure onset, EEG features, intellectual disability level, type of mutation, absence vs presence of non-epileptic paroxysmal events, and neuroimaging features. The percentage of patients with epilepsy was higher compared to what had previously been reported (64% vs 16-50%), which is likely explained by the larger size of the sample and longer follow-up compared to previous studies. We classified our patients into three groups based on their electroclinical features, long-term outcome and response to therapy: group 1 with developmental and epileptic encephalopathy, group 2 with mild epileptic phenotype, group 3 epilepsy-free. The neurocognitive impairment and regression occurring in patients with severe epilepsy may be related to cortical dysplasia and hippocampal sclerosis, which likely are genetically determined and in turn play a role in the epileptogenic process. Finally, a genotype-phenotype correlation linking the presence/severity of epilepsy to the nature of the structural/functional consequences of mutations was observed, indicating that mutations affecting residues located within or closed to the CR2 surface of BRAF interacting with the inhibitory 14-3-3 protein are generally associated with a milder expression of disease or with a condition not including epilepsy as an associated feature.

Behavioral phenotypes across the RASopathies	Marie-Maude Geoffray[1,2], Bruno Falissard[3], Jonathan Green[2,4,5], Bronwyn Kerr[4,6], D. Gareth Evans[2,4,7], Susan Huson[4,6], Emma Burkitt-Wright[4,6], Shruti Garg[2,4,5]

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This presentation describes the social behavioural phenotype in the RASopathies focusing on Noonan and CFC syndromes. Here we report an overview of the biological mechanisms underpinning the social communication deficits in the RASopathies, and the evidence from behavioural phenotypic studies. The presentation concludes with findings from our recently published work (https://doi.org/10.3389/fpsyt.2020.585700) comparing early neurodevelopment and the social behavioural phenotype in Neurofibromatosis 1, Noonan syndrome and CFC.

SESSION 5: Clinical Manifestations
Moderator: Jeroen den Hertog PhD, Hubrecht Institute, The Netherlands

Mosaicism in RASopathies: HRAS in cancer and Costello syndrome	Karen Gripp MD, Nemours/DuPont Hospital, Wilmington, DE

Specific missense mutations in HRAS, typically arising in the paternal germline, are seen in individuals with Costello syndrome. Individuals with Costello syndrome are at increased risk for embryonal rhabdomyosarcoma, neuroblastoma and early onset bladder cancer. This tumor risk is ~15% by age 20 years and depends upon the amino acid affected by the pathogenic variant. In embryonal

rhabdomyosarcomas in Costello syndrome, uniparental disomy for the mutated paternal HRAS allele with loss of the maternal wildtype allele has consistently been demonstrated. This UPD affects imprinted genes on 11p.

Somatic HRAS missense variants are identified in isolated malignancies unrelated to Costello syndrome. Mutation hotspots for oncogenic variants in isolated malignancies show some overlap with those in Costello syndrome. Variants resulting in the strongest effect on the RAS/MAPK signaling pathway may be frequent in isolated malignancies, but, due to their severe disturbance of this growth regulatory pathway, may not be compatible with life in a heterozygous individual.

When an HRAS pathogenic variant arises postzygotically during embryonic development, the variant is expected to be present in some, but not all, tissues of the individual. Such mosaicism can result in an individual with variable findings, depending upon the affected tissues. Schimmelpenning syndrome is one example of such a phenotype, combining ectodermal and additional tissue involvement. Rarely, individuals with ectodermal findings suggestive of an HRAS variant and a malignancy typical for Costello syndrome, such as rhabdomyosarcoma or early onset bladder cancer, have been identified to carry such mosaic HRAS variants. In contrast to the typically paternally derived HRAS variants in Costello syndrome, in postzygotically acquired HRAS mosaicism, the maternal and paternal alleles are equally likely affected. Thus, presentation of postzygotic HRAS variants may depend not only on the specific variant and the affected tissues, but malignancy risk may also vary upon which allele is mutated.

Modeling skeletal myopathy in CFC syndrome

Katherine A. Rauen, Yoshiko Maeda, Bradley P. Ander, Catrin A. Pritchard, William E. Tidyman

Cardio-facio-cutaneous syndrome (CFC) is a human multiple congenital anomaly syndrome that is caused by activating heterozygous mutations in either BRAF, MEK1, MEK2 or KRAS. CFC belongs to a group of syndromes known as RASopathies wherein individuals typically have characteristic dysmorphic features, cardiac defects, ectodermal anomalies and developmental delay. Skeletal muscle hypotonia is a ubiquitous phenotype of RASopathies, especially in CFC syndrome. To better understand the underlying mechanisms for the skeletal myopathy in CFC, a mouse model with an intermediate activating $Braf^{L597V}$ variant was used in this study. The activating $Braf^{L597V}$ allele resulted in phenotypic alterations in skeletal muscle characterized by a reduction in fiber size which led to a reduction in muscle size which are functionally weaker. Ras/Mitogen-activated protein kinase (MAPK) pathway activation caused inhibition of myofiber differentiation during embryonic myogenesis and global transcriptional dysregulation of developmental pathways. We determined that in our model, inhibition in differentiation can be rescued by MEK inhibition. In summary, a skeletal myopathy was identified in the CFC $Braf^{L597V}$ mouse validating the use of models to study the effect of Ras/MAPK dysregulation on skeletal myogenesis. RASopathies present a novel opportunity to identify new paradigms of myogenesis and further our understanding of Ras in development. Rescue of the phenotype by inhibitors may help advance the development of therapeutic options for RASopathy patients.

Skin and hair manifestations in Costello and CFC syndromes

Maija Kiuru MD PhD, UC Davis, CA

Skin and hair findings are important in making a clinical diagnosis of a RASopathy. However, phenotypical overlap exists, particularly between cardio-facio-cutaneous syndrome (CFC), Costello syndrome (CS) and Noonan syndrome. Absent or sparse eyebrows, keratosis pilaris and numerous melanocytic nevi are characteristic of CFC, while cutaneous papillomas and full eyebrows are more common in CS. Additionally, in CFC hair shafts are darker and thicker, while in CS, synophrys and trichomegaly is common. Furthermore, individuals with CS caused by an HRAS mutation other than p.G12S are more likely to present with straight hair. The number of melanocytic nevi in CFC is significantly increased throughout the body and increases in adolescence. The risk of melanoma is unknown in CFC, but protection from UV light and regular skin exams is recommended.

Ocular manifestations in RASopathies

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The important role of Ras pathway in ocular development has been shown in several animal models such as drosophila, xenopus and mouse models as well as in invitro studies[1-3]. Ocular features have also been reported in individuals with RASopathies[4-7]. Here, we report on comprehensive eye findings in Costello, Cardiofaciocutaneous and Noonan syndromes based on retrospective medical record analysis and cross-sectional studies in patient support group conferences to obtain this data. The most common presenting features included blurred vision, problems with stereopsis, and photophobia. On exam and chart review, the majority of individuals with all three syndromes had the following ocular findings: strabismus, refractive errors, nystagmus, ptosis and optic nerve anomalies. Rarely, additional features such as keratoconus, prominent corneal nerves, retinal dystrophies, delayed visual maturation and cortical visual impairment were noted. Several clinical features that cause vision impairment such as refractive errors, strabismus, nystagmus, and ptosis are amenable to correction using glasses and surgery and can present in infancy. Hence, pediatric ophthalmology evaluation at

the time of diagnosis with routine biannual or annual follow up is recommended to prevent amblyopia, facilitate optimal vision development, and improve quality of life for these individuals.

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SESSION 6: Selected Junior Investigator Presentations

Moderators: Amy Roberts MD, Boston Children's, MA; William Timmer PhD, NCI CTEP, Bethesda, MD

Junior Investigator Finalist Presentation #1: **A Collection of Drosophila Models to Identify RASopathy Subtype Specific Differences**	Tirtha Das PhD, Mount Sinai, NYC, NY

Animal models and clinical data have vastly improved our understanding of gene variants that lead to RASopathies. Still, understanding the full spectrum of cell signaling and cell biological changes induced by these variants and its effects on tissue development and the eventual pathology in patients remains a key focus of current research.

Drosophila has been a powerful genetic system with which to decipher fundamental cell and developmental biology questions, and it includes discovery of components of the RAS/MAPK pathway. To gain a more comprehensive understanding of how variants in genes encoding RAS/MAPK components are associated with RASopathies, we initially developed 13 Drosophila transgenic lines. Each fly line expresses a different human disease isoform associated with a RASopathy. Using these models, we developed a platform that consisted of evaluating overall tissue phenotypes, monitoring signaling pathway activity, and evaluating response to potential therapeutics. We showed that, similar to their human counterparts, each Drosophila line has similarities but also important phenotypic differences including regulation of signaling pathways. Assessing wing vein development, which is directly controlled by MAPK signaling, we found that expressing RAF1L613V variant throughout the entire developing wing tissue promoted excess wing vein formation, supporting a gain-of function effect of this variant. In contrast, similar expression of RAF1D486G inhibited wing vein formation suggesting a potential dominant negative effect of this variant. We found that some of these variants also activate pathways outside the core RAS signaling pathway, including the Hippo and SAPK/JNK signaling networks. In our drug screens, we identified two classes of clinically relevant drugs, statins and histone deacetylase inhibitors, that improved viability across most RASopathy lines. In contrast, several canonical RAS pathway inhibitors proved poorly effective against, e.g., SHP2-expressing lines encoded by PTPN11. Our analyses identified differences in tissue phenotypes, in activation of signaling pathways, in biomarkers of disease progression, and candidate drugs showing efficacy that can be further pursued as candidate therapeutics.

Currently, we have expanded our analysis to further improve our understanding of the different RASopathy variants. First, we have developed 20 additional models to include variants of genes encoding MEK, RIT, SOS, SHOC. Second, we are combining the CRIMIC technology with the GAL4/UAS system to allow temporal and spatial expression of human wild-type and RASopathy variant genes at endogenous physiological levels, and importantly, at a clinically relevant gene dosage. This technology also tests if variants can rescue flies to adult stages, in the absence of the endogenous gene function. Using this approach, we find that RAF1D486G variant can rescue a small percentage of flies (2-5%) to adult stages in the absence of endogenous Raf function. Taken together with our previous work, we postulate that RAF1D486G, previously described as a loss-of-function variant, can have a more complex function. It can directly or indirectly activate the RAS/MAPK pathway itself to rescue loss of RAS/MAPK activity and in certain contexts also have a dominant negative effect on the same pathway (wing). Finally, to assess activation of signaling pathways at the nuclear/transcriptional level, we crossed these variants to fly lines with well-established pathway specific lacZ reporters, which encompass most of the major signaling pathways in Drosophila. Using this assay, we find that the RAF1L613V variant can activate transcriptional reporters of EGFR, Hippo,

and FGFR signaling, some of which have not previously been described. Activation of the Hippo pathway by the RAF1L613V variant has been previously described, further validating our approach of using this strategy to uncover novel signaling pathway activity.

Our analysis, using 33 Drosophila lines, has provided a broad overview of how RASopathy variants alter signaling in tissues and in some cases uncovered new mechanisms of action. In the future, it could provide valuable insights into how pathogenic variants promote specific traits in humans as well as enable tailored therapies for them.

Junior Investigator Finalist Presentation #2: **Abnormal Myelin Differentially Impairs Learning in Models of the RASopathies Neurofibromatosis Type 1 and Costello Syndrome**	Alejandro Lopez-Juarez PhD, UT Rio Grande Valley, TX

Abnormal brain white matter and myelin are common features in the RASopathies Neurofibromatosis Type 1 (NF1) and Costello Syndrome (CS). The potential contribution of these abnormalities to the neurological issues in NF1 and CS have been discussed for a long time; however, experimental evidence for or against this idea is very limited. In his presentation, Dr. Lopez-Juarez reveals defects in acquisition of motor skills in myelin-focused mouse models of NF1 and CS. In both models, learning curves of fine motor skills, in a myelin-regulated test (the Complex Wheel; CW), are subnormal. Interestingly, different factors underlie learning deficiencies in NF1 as compared to CS mice. By connecting these findings with previous evidence, differential impact of defective myelin structure on elements influencing learning in NF1 and CS is proposed.

Junior Investigator Finalist Presentation #3: **Biochemical analysis of RIT1 reveals preferential interaction with RAF1 and provides evidence for therapeutic intervention of cardiac hypertrophy**	Richard Van BS, UCSF, CA, and Morgan Wagner BS, NCI RAS Initiative, Frederick, MD

RASopathies are a group of genetic disorders caused by germline mutations in the components of the RAS/RAF/MAPK pathway. Mutations within the small GTPase RIT1 have been associated with Noonan syndrome (NS), the most common RASopathy, and is coincident with a high prevalence (~54%) of hypertrophic cardiomyopathy; however, the mechanisms that underly RIT1-mediated MAPK activation remain understudied. Plasma membrane (PM) association of RAS/small GTPases occurs via prenylation of their C-terminal hypervariable region (HVR) and is essential for their activity, RAF interaction, and downstream MAPK activation. In contrast to most RAS GTPases, RIT1 does not undergo canonical prenylation within its HVR, yet it is often localized at the PM. In this study, we utilized biophysical, biochemical, and cell biological techniques to characterize how RIT1 interacts with the PM and its RAF effectors. Confocal imaging of GFP-RIT1 revealed that membrane localization is dependent on basic and hydrophobic residues in the HVR. Compared to canonical RAS GTPases, RIT1 weakly interacted with RAF effectors. The common NS RIT1 mutant A57G exhibited increased affinity to RAF proteins and preferentially interacted with RAF1. In addition, we demonstrate that membrane localization is required for this functional interaction. Despite the increased interaction with RAF proteins, RIT1 A57G was unable to activate the downstream MAPK pathway in the absence of the canonical RAS GTPases. Given the high incidence of cardiac complications in the RIT1 mutant NS population, we have also assessed the therapeutic benefit of inhibiting MEK kinase in our RIT1M90I NS mouse model. Treatment with low dose trametinib alleviated cardiac hypertrophy, providing evidence for the significance of MAPK activation in this context. Understanding how RIT1 engages with its effectors may provide further insights into the role that RIT1 plays in NS.

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