



International Meeting on Genetic Syndromes of the
Ras/MapK Pathway
“Finding Our Way Back to the Bedside”

July 29 – 31, 2011

Westin O’Hare

Chicago, IL

**International Meeting on Genetic Syndromes of the Ras/MAPK Pathway
“Finding Our Way Back to the Bedside”**

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We thank the following organizations for their generous sponsorship of this meeting:

International Costello Syndrome Support Group (ICSSG)



Costello Syndrome Family Network (CSFN)



THE NOONAN SYNDROME SUPPORT GROUP, Inc.

Forging a path to improve lives through family support, research and education.



CFC international

Cardio-Facio-Cutaneous Syndrome



Symposium Program

Friday July 29th

8:00 - 10:00 pm Dessert and Poster Session
(Symposium attendees and advocacy/family groups)
Goals: To encourage collaboration, participants, including the families from the advocacy groups, are invited to the poster session. This forum is a unique opportunity for researchers and clinicians to interact with families affected by Ras/MAPK pathway syndromes in a non-clinical setting. In addition, it makes families aware of research, in which they may become involved. We are expecting about 300 people to attend the gathering.

Saturday July 30th

7:00 - 8:15 am Breakfast

8:15 - 8:30 am **Welcoming Comments and Introduction** – Bruce Gelb, Amy Roberts and Lisa Schoyer

8:30 - 9:00 am **Keynote Presentation:** Leslie Gordon, MD, PhD
Lessons Learned on the Path to Developing Treatment for Progeria

9:00 - 9:30 am **Advocates' Panel**
The keynote address will be a presentation by Leslie Gordon, MD, PhD, a pediatrician who leads the Progeria Research Foundation, which has spearheaded research leading to clinical trials. The advocates' panel will feature all four groups and highlight living with a person affected with a Ras/MAPK pathway syndrome in a questions-and-answers format to promote communication between the families and the professionals.

9:30 - 10:50 am **Gene Discoveries- Recent and Future**
Moderator: Ineke van der Burgt, MD
Goals: To present the most recent information about novel genes discovered to cause Ras/MAPK pathway disorders, including available information about the biochemical effects of the proteins and signal transduction, as well as a discussion about the use of next generation sequencing to drive current gene discovery efforts

9:30 - 9:50 am	CBL	Hélène Cavé, PhD
9:50 - 10:10 am	NRAS	Martin Zenker, MD
10:10 - 10:30 am	SHOC2	Marco Tartaglia, PhD
10:30 - 10:50 am	Exome and Genome Sequencing	Joep de Ligt, PhD

10:50 - 11:10 am Break

11:10 - 12:30 pm **Advances in Clinical Care**
Moderators: Karen Gripp, MD and Kate Rauen, MD, PhD
Goals: To present recent advances in clinical care, including those driven by molecular discoveries of the Ras/MAPK disorders. Efforts to develop evidence-based approaches and organized networks to support future clinical research will be emphasized. The final presentation describes ongoing studies about the effects of various Ras/MAPK mutations on neurocognitive development, a likely target for future therapeutic intervention

11:10 - 11:30 am Genotype-Phenotype Amy Roberts, MD

Saturday July 30th, Continued

11:30 – 11:50 am	Epidemiological Features of Costello and CFC Syndromes	Yoko Aoki, MD, PhD
11:50 - 12:10 pm	Clinical Pathways: The Dysceme Experience	Bronwyn Kerr, MD
12:10 - 12:30 pm	Neurodevelopmental Profiles for RASopathies	Rene Pierpont, PhD
12-30 - 1:15 pm	Lunch Break	
1:15 - 2:00 pm	Young Investigator Competition	
	Moderators: Amy Roberts, MD and Bruce D. Gelb, MD	
	Judges: Judith Allanson, MD, Helene Cave, PhD, and Leslie Gordon, MD, PhD	
	Goals: To provide a forum for trainees to present their outstanding research in order to encourage their participation in this meeting as well as to present the most recent research information for our attendees.	
2:00 - 3:20 pm	Ras Pathway Biology	
	Moderator: Marco Tartaglia, PhD	
	Goals: To present fundamental information about the biology of Ras/MAPK signal transduction as it relates to specific aspects of human biology perturbed in patients with disorders of this pathway. Emphasis will be on four aspects: circadian rhythm/sleep, cardiogenesis and cardiac muscle biology, hematopoiesis/cancer, and growth hormone signaling – that may represent targets for future targeted therapeutic interventions.	
2:00 - 2:20 pm	Circadian Rhythm and Sleep	Amita Sehgal, PhD
2:20 - 2:40 pm	Skeletal Muscle Pathology in Costello and CFC syndromes	Katherine Rauen, MD, PhD
2:40 - 3:00 pm	Hematopoiesis and Cancer	Rebecca Chan, MD, PhD
3:00 - 3:20 pm	Growth Hormone Signaling	Jessica Schwartz, PhD
3:20 - 3:40 pm	Break	
3:40 - 5:20 pm	Cell and Animal Models of Disease	
	Moderators: Benjamin G. Neel, MD, PhD and Marco Tartaglia, PhD	
	Goals: To discuss the elucidation the pathogenesis of critical aspects of the Ras/MAPK disorders from modeling in mice engineered to have relevant disease-causing point mutations as well as in induced pluripotent stem cells derived from patients with NS and LS. These presentations will include information about how these model systems might be used to obtain pre-clinical data needed to support clinical trials. The final speaker will discuss the use of fruit fly models of disease, including NS, for high throughput drug screening.	
3:40 - 4:00 pm	Cardiac Defects in Ras Pathway Mouse Models	Benjamin G. Neel, MD, PhD
4:00 - 4:20 pm	LEOPARD Mouse Model: Phenotype and Rapamycin Therapy	Maria Kontaridis, PhD
4:20 – 4:40 pm	Neurodevelopment in Noonan Syndrome Mice	Alcino J. Silva, PhD
4:40 - 5:00 pm	Induced Pluripotent Stem Cells	Bruce D. Gelb, MD
5:00 - 5:20 pm	Drug Screening with Drosophila	Ross Cagan, PhD
6:30 - 8:00 pm	Banquet Dinner for symposium registrants	

Sunday July 31st

7:00 - 8:00 am	Breakfast	
8:00 - 9:20 am	Clinical Trials Moderators: Leslie Gordon, MD, PhD and Martin Zenker, MD Goals: Drugs that might be efficacious for the Ras/MAPK pathway disorders are at various stages- FDA- approved drugs such as statins are in clinical trials for NF1; novel drugs are being developed and tested for cancer; melatonin has been trialed successfully for sleep disturbances in children. Speakers will provide information about drug developmental pipelines and clinical trials. The final speaker will discuss a recent NS clinical trial directed at augmenting height, providing important lessons for all Ras/MAPK pathway disorders.	
8:00 - 8:20 am	Statin Therapy for Neurofibromatosis Type I	Maria T. Acosta, MD
8:20 - 8:40 am	Ras Pathway Inhibitors for NF1 and Cancer	D. Wade Clapp, MD
8:40 - 9:00 am	Sleep Disturbance and its Treatment in Children with RASopathies	Giacomo Della Marca, MD, PhD
9:00 - 9:20 am	NSEuroNet and the Gendia Mutation Database	Martin Zenker, MD
9:20 - 9:50 am	Advocates Panel Discussion	
9:50 - 10:00 am	Charging the Working Groups	
10:00 - 10:15 am	Break	
10:15 - 11:30 am	Working Group Meetings A) Preclinical Consortium (Leaders: Benjamin Neel, MD and Alcino Silva, PhD) Organize development and characterization of cell and animal models including pre-clinical drug testing B) RASopathies Resource Network (Leaders: Bruce D. Gelb, MD, Marco Tartaglia, PhD, Martin Zenker, MD and Lisa Schoyer) Facilitate collaborative gene discovery, genotype-phenotype assessments, mutation informatics C) Clinical Network (Leaders: Judith Allanson, MD, and Katherine A. Rauen, MD, PhD) Organize diagnostic criteria, best clinical practices, centers of excellence and natural history studies D) Clinical Trials Consortium (Leader: Amy Roberts, MD) Determine the endpoints, candidate therapies, and mechanism of support for future clinical trials.	
11:30 am– 12:30 pm	Reports from the Working Groups and Final Discussion	

KEYNOTE SPEAKER



Leslie B. Gordon, MD, PhD

Dr. Gordon is an Associate Professor of Pediatrics Research at Warren Alpert Medical School of Brown University and Hasbro Children's Hospital in Providence, R.I., where she conducts her basic science research on HGPS. She is a staff scientist at Children's Hospital Boston in the department of Anesthesia and Harvard University Medical School, where she conducts her clinical research on Progeria as co-chair for clinical treatment trials for children with Progeria. Dr. Gordon is co-founder of The Progeria Research Foundation (PRF) and serves as the organization's volunteer Medical Director. Dr. Gordon is the Principal Investigator on three ongoing PRF programs for Progeria, including a medical and research database, cell and tissue bank, and the genetic diagnostics program. She has organized 6 NIH-funded, international scientific meetings on Progeria. She has received the March of Dimes Basil O'Connor Award, the American Heart Association Scientist Development Award, The Gerontological Society of America Award for contributions to Progeria, a NIH Bench to Bedside Grant, and the Mother of the Year award from Working Mother Magazine.

SPEAKER ABSTRACTS

Progeria, aging and translational medicine: from obscurity to treatment trials and beyond

Leslie B. Gordon, MD, PHD

Hutchinson-Gilford progeria syndrome (Progeria) is a rare segmental premature aging syndrome that affects 200-250 children worldwide at any one time. It is caused by a mutation in the *LMNA* gene, whose normal lamin A protein product is central to nuclear structure and function in differentiated cells. The global expression of lamin A, and hence the aberrant protein produced in Progeria called progerin, results in a multisystem disease. Children with Progeria die of global, accelerated atherosclerosis at an average age of 13 years.

Overall, The Progeria story is not unlike that of the RASopathies, or many other rare genetic diseases whose affected populations could benefit from exponential increases in attention. This session is intended to relate the story of how the field of Progeria has traveled from practical obscurity towards the center of what we hope will be a translational medicine success story. The story of Progeria demonstrates the absolute need for a multipronged approach to solving the most challenging of life's problems – curing a rare genetic disease.

Germline mutations of the *CBL* gene : a new *RAS*opathy with predisposition to juvenile myelomonocytic leukemia (JMML)

Hélène Cavé, PharmD, PhD

Genetics department, CHU Robert Debré and INSERM U940, University institute of Haematology, Paris, France

CBL, an E3 ubiquitin ligase and multi adaptor protein, controls proliferative networks by downregulating the growth factor receptor signaling cascades in various cell types. *CBL* missense mutations have recently been found in 10-15% patients having juvenile myelomonocytic leukemia (JMML), an aggressive myelodysplastic and myeloproliferative neoplasm of early childhood. The majority of these patients displays germline heterozygous *CBL* mutations. The process of tumorigenesis is in line with the classical Knudson hypothesis for tumor suppressor genes. The germline mutation, inherited from the parents in about half of cases, represents the first hit. Somatic loss of heterozygosity of 11q23.3, due to acquired somatic uniparental isodisomy is the second hit, positively selected for in JMML cells. Noteworthy, a mutation targeting Y371, is found in about half of the patients with germline *CBL* mutation who develop JMML.

Besides JMML predisposition, germline *CBL* mutation defines a new dominant genetic condition with a markedly variable phenotype combining dysmorphic features, hyperpigmented skin lesions, cryptorchidism, cardiopathy, neurological lesions, developmental delay, postnatal growth retardation, and autoimmune phenomena. Some clinical features are reminiscent of Noonan syndrome (NS) or type I-neurofibromatosis (NFI), and *CBL* mutations are found in about 1% of patients suspects of NS. However, the “*CBL* syndrome” only partially overlaps with NS and probably remains largely underdiagnosed in patients who do not display hematological features. Further studies will permit to refine the clinical description of patients with this syndrome and to better appreciate the risk of leukemia or other malignancies associated with it.

1. Pérez B, et al. Germline mutations of the *CBL* gene define a new genetic syndrome with predisposition to juvenile myelomonocytic leukaemia. *J Med Genet.* 2010 Oct;47(10):686-91. Epub 2010 Jun 12.
2. Martinelli S, et al. Heterozygous germline mutations in the *CBL* tumor-suppressor gene cause a Noonan syndrome-like phenotype. *Am J Hum Genet.* 2010 Aug 13;87(2):250-7. Epub 2010 Jul 8.
3. Niemeyer CM, et al. Germline *CBL* mutations cause developmental abnormalities and predispose to juvenile myelomonocytic leukemia. *Nat Genet.* 2010 Sep;42(9):794-800. Epub 2010 Aug 8.

NRAS mutations – a rare cause of Noonan syndrome

Martin Zenker

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Noonan syndrome (MIM163950) is a clinically recognizable and genetically heterogeneous disorder. The common denominator of the various genetic alterations found in patients with Noonan syndrome and related disorders is that they cause dysregulation of RAS-MAPK signaling. Even if strict clinical criteria are applied, the molecular genetic lesion currently remains unidentified in about 10-20% of cases, thus suggesting additional genes for Noonan syndrome. An international research collaboration has recently led to the discovery of NRAS mutations in 4 unrelated patients out of a large cohort of over 900 individuals negative for previously known mutations with a phenotype fitting or suggestive of Noonan syndrome (Cirstea et al., Nat Genet 2010). A few additional cases have been observed since then. The current data suggest that NRAS mutations account for less than 1% of cases with Noonan syndrome. The phenotype associated with NRAS mutations appears to have nothing specific and falls into the clinical category of Noonan syndrome with a relatively mild clinical expression in some of the mutation carriers. Although the number of mutation-positive cases is still too low to define valid genotype-phenotype correlations, it is notable that the typical congenital heart defects, such as pulmonary stenosis, hypertrophic cardiomyopathy and septal defects, were only observed in one third of these patients.

Three Noonan syndrome-associated NRAS mutations. I24N, T50I, and G60E, have been published, so far. Similar to KRAS, NRAS germline mutations are not identical to those commonly observed as somatic lesions in cancer. They have been shown to cause RAS overactivation. The precise mechanisms by which specific mutant NRAS proteins cause developmental anomalies typical of Noonan syndrome are still poorly understood.

Full list of authors: Ion C Cirstea, Kerstin Kutsche, Radovan Dvorsky, Lothar Gremer, Claudio Carta, Denise Horn, Amy E Roberts, Francesca Lepri, Torsten Merbitz-Zahradnik, Rainer König, Christian P Kratz, Francesca Pantaleoni, Maria L Dentici, Victoria A Joshi, Raju S Kucherlapati, Laura Mazzanti, Stefan Mundlos, Michael A Patton, Margherita Cirillo Silengo, Cesare Rossi, Giuseppe Zampino, Cristina Digilio, Liborio Stuppia, Eva Seemanova, Len A Pennacchio, Bruce D Gelb, Bruno Dallapiccola, Alfred Wittinghofer, Mohammad R Ahmadian, Marco Tartaglia & Martin Zenker

Ras/MAPK Disorders: Genotype Phenotype Correlations

Amy E. Roberts, M.D.

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Noonan syndrome (NS) is a genetic multisystem disorder characterized by recognizable facial features, developmental delay, learning issues, short stature, congenital heart disease, renal anomalies, lymphatic malformations, and/or bleeding problems. NS-causing mutations alter genes encoding proteins with roles in the Ras/mitogen-activated protein kinase (Ras/MAPK) pathway, leading to pathway dysregulation. Thus far, eight genes have been shown to cause NS or closely related conditions (*PTPN11*, *SOS1*, *KRAS*, *NRAS*, *RAF1*, *BRAF*, *SHOC2*, and *CBL*). There are multiple clinically relevant genotype-phenotype correlations that aid in risk assessment and patient management. Genotype phenotype correlation with regard to cardiovascular, growth, development, cutaneous, and hematological features will be discussed.

Features of Cardiofaciocutaneous (CFC) syndrome include multiple congenital anomalies and intellectual disabilities, failure to thrive and short stature, congenital heart defects, and a characteristic facial appearance. The phenotypic presentation of CFC significantly overlaps that of NS and mutations in four Ras/MAPK genes, *BRAF*, *KRAS*, *MEK1*, and *MEK2*, have been found to cause CFC.

The dysmorphic facial features, skin abnormalities, short stature, and cardiac abnormalities in Costello syndrome (CS) have prompted comparison to NS and CFC. Mutations in the Ras/MAPK gene *HRAS* have been identified as the cause of approximately 85% of cases of CS.

Genotype phenotype correlations, as they relate to Cardiofaciocutaneous (CFC) syndrome and Costello syndrome (CS) will be reviewed. Detailed assessment of the phenotype may be helpful in guiding molecular genetic diagnostic testing for an individual.

Epidemiological Features of Costello and CFC Syndromes

Yoko Aoki and Yoichi Matsubara

Department of Medical Genetics, Tohoku University School of Medicine, Sendai, Japan

Only a few Japanese patients with Costello and CFC syndromes had been reported by 2005. The number of patients is growing after the identification of causative genes for these disorders. However, clinico-epidemiological features of these disorders remain to be elucidated. In order to assess the prevalence, natural history, prognosis and tumor incidence, we conducted a nationwide prevalence study of patients with Costello and CFC syndromes in Japan. The protocol we followed has been established by study groups on intractable diseases granted by the Ministry of Health and Welfare of Japan. The prevalence of intractable diseases, including moyamoya disease, pancreatitis and sudden deafness has been reported using the same protocol. The study consisted of a two-stage questionnaire survey. The first-stage survey inquired the number of mutation-positive patients as well as clinically suspected patients. The second-stage survey asked for detailed clinicoepidemiological information of each patient reported. The estimated number of patients, prevalence, clinical manifestations of mutation-positive patients and activities of daily living were clarified in this study. Evaluation of fifteen adult patients aged 18-32 years revealed that twelve patients had moderate to severe mental retardation, but eleven of them live at home and ten can walk independently, suggesting that the number of adult patients were underestimated. This is the first epidemiological study of both disorders. Identification of patients older than 32 years of age and follow up of the reported patients in the current study will be important to estimate the precise prevalence and natural history of these disorders.

Clinical pathways: the DYSCERNE experience

Bronwyn Kerr, M.B.B.

University of Manchester, Manchester, UK

Establishing best practice guidelines for rare disorders is difficult. Literature series are often small, and may be biased towards the most severely affected. Information on the natural history of rare disorders over a lifetime is usually incomplete. Reports of treatments and interventions will usually involve only small numbers of patients and short-term follow-up.

For affected people and their families, a related issue is the provision of available information in formats that are useful to both affected individuals and their non-expert care givers.

The DYSCERNE Network of Centres of Expertise in Dysmorphology (www.dyscerne.org) project was funded by the EU (2007-2009). One of the project aims was development of management guidelines for selected rare syndromes. Guidelines included; criteria for diagnosis, information on clinical management at different life stages, and when specialist referral is needed.

Guideline development utilised a modified SIGN (Scottish Intercollegiate Guidelines Network) methodology. The process and outcome will be presented, with particular reference to Neurofibromatosis type I and Noonan syndrome.

Neurodevelopmental Profiles for RASopathies

Rene Pierpont, Ph.D.

The last few decades have seen rapid progress toward establishing neuropsychological profiles associated with Ras/MAPK pathway syndromes. Among affected individuals, cognitive functioning can vary from significant global intellectual disability to mild delays in specific domains. In a subset of individuals, no significant learning or behavior problems are seen. Research using animal models has begun to reveal neurobiological sources of differences in learning and memory processes in the RASopathies, as well as suggest potential methods to circumvent these differences. However, additional research is needed to identify the most useful neurocognitive targets of intervention in humans. Development of effective educational or pharmacological mechanisms that will impact long-term outcomes depends on progress in a number of areas. In particular, it will be critical to identify key individual characteristics that can predict response to intervention in any given domain. While current research has established that variation in intellectual functioning and adaptive skills can be partially explained by the differences in genotype, wide variability in outcomes can be observed even among people with the same mutations. Additional important characteristics associated with treatment response may include chronological age at intervention, presence of medical risk factors, and baseline level of functioning. Further, neuropsychological measurement instruments for clinical trials must be validated within the target population and evaluated for reliability and practice effects. As research in this area progresses, there is great promise to positively impact the lives of individuals with genetic syndromes of the Ras/MAPK pathway.

N-myristoylation of SHOC2 and Mazzanti syndrome

Marco Tartaglia

Cordeddu and co-workers (Nat. Genet. 2009, 41:1022-1026) reported that the invariant c.4A>G missense (p.Ser2Gly) change in the *SHOC2* gene, which encodes a widely expressed non-membranous protein that positively modulates RAS-MAPK signal flow, underlies a distinctive RASopathy previously recognized as Noonan-like syndrome with loose anagen hair [OMIM 607721] by Mazzanti and colleagues (Am. J. Med. Genet. 2003, 118A:279-286). This mutation was demonstrated to promote N-myristoylation of the protein and its aberrant targeting to the plasma membrane. N-myristoylation is an irreversible cotranslational lipid modification, and is observed to occur in many signal transducers that require to be anchored to the cytoplasmic leaflet of cell membranes to carry out their function.

SHOC2 is composed almost entirely by leucine-rich repeats (LRR), and has a KEKE motifs-rich sequence at the N-terminus. Both regions provide a structural framework for protein-protein interactions, and the protein is believed to function as a scaffold linking RAS to downstream signal transducers. First functional characterization of the mutant protein indicates that the expression of *SHOC2*^{S2G} enhances ERK activation in a cell type-specific fashion. The observations that the subcellular localization of *SHOC2* is restricted to the nucleus following EGF stimulation and that such translocation is impaired in the mutated protein, however, suggest that the disease-causing mutant might exert a wider perturbing effect on intracellular signaling.

Subjects heterozygous for the c.4A>G change share a phenotype that is characterized by an unusual combination of features occurring in other RASopathies. The phenotype of these subjects is notable for the reduced growth often associated with GH deficiency, cognitive deficits, distinctive hyperactive behaviour, and peculiar hair anomalies (*i.e.*, loose anagen hair). Most affected individuals exhibit hairless and darkly pigmented skin with eczema or ichthyosis. Ectodermal anomalies also include sparse eyebrows and dystrophic/thin nails. The voice is characteristically hoarse or hypernasal. Mitral valve dysplasia and septal defects are significantly overrepresented compared with other RASopathies.

Based on the specific underlying molecular basis and the homogeneous and distinctive clinical phenotype, this disorder appears to represent a discrete nosologic entity, for which the eponymous name of Mazzanti syndrome is suggested.

Role of Neurofibromatosis/Ras in circadian rhythms and sleep

Amita Sehgal

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Sleep is controlled by a homeostatic system that drives the need to sleep and a circadian system that imposes a ~24hour rhythm on sleep and wake. Ras-MAPK signaling has been implicated in both these systems in multiple species. Using a *Drosophila* model, we found several years ago that loss of the *Neurofibromatosis 1* gene results in loss of circadian rhythms of sleep:wake. We showed also that the mutant phenotype results from increases in Ras-MAPK signaling, which was the first demonstration that *Drosophila* NFI signals through Ras-MAPK. Because the central clock was unaffected in NFI mutants we attributed the arrhythmic phenotype to an effect on output from the clock. Other studies, done in mammals and fungi, supported a role for MAPK signaling in circadian output and mammalian work was indicated a function in light input to the clock. However, recent work indicates that NFI may also directly affect sleep. Following up on our *Drosophila* work, Johnson et al¹ found that children afflicted with NFI have sleep disturbances. While it can be difficult to distinguish between circadian and homeostatic influences on sleep in patients, our recent fly work also indicates an effect of NFI on the homeostatic control of sleep.

¹Johnson et al, Psychological disturbance and sleep disorders in children with neurofibromatosis type I, [Dev Med Child Neurol](#). 47:237-42 (2005).

Skeletal muscle pathology in Costello and cardio-facio-cutaneous syndromes: Developmental consequences of germline Ras/MAPK activation on myogenesis

Katherine A. Rauen, William E. Tidyman, Han S. Lee

University of California San Francisco, San Francisco, California

Cardio-facio-cutaneous syndrome (CFC) and Costello syndrome (CS) are two of the more rare RASopathies caused by altered signal transduction of the Ras/mitogen-activated protein kinase (MAPK) pathway. All of the RASopathies exhibit some degree of hypotonia, but CS and CFC are more severe. To determine if individuals with CS and CFC have an underlying skeletal myopathy, we systematically evaluated skeletal muscle pathology in both conditions. We reviewed pathology reports from 6 individuals who had undergone a skeletal muscle biopsy, and we reviewed histology slides on 2 cases with CS and 1 case with CFC. All patients in the cohort had histopathologic findings, and two consistent abnormalities were identified. The first was the presence of abnormal muscle fiber size and variability, and the second was the presence of type 2 fiber predominance. Given the degree of hypotonia typically present in these patients, the overall architecture of the muscle was relatively normal, without showing indications of severe structural histopathology or metabolic abnormalities. Because the Ras/MAPK pathway is vital for skeletal myogenesis, we evaluated the effects of CS and CFC mutations on myogenesis using C2C12 myoblasts. All CS/CFC mutations inhibited myoblast differentiation as indicated by diminished myosin heavy chain expressing cells and a decrease in the number of myotubes as compared to controls. These findings indicate that CS and CFC may have a true myopathy related to an inherent dysregulation of skeletal myogenesis, which further expands our understanding of the consequences of germline Ras/MAPK mutations.

Ras Pathway Biology: Hematopoiesis and Cancer

Rebecca J. Chan

Germline gain-of-function mutations within genes contributing to the Ras-MAPK pathway have been identified in individuals with the phenotypically overlapping congenital disorders, Noonan syndrome, Costello syndrome, Cardio-facio-cutaneous syndrome, and LEOPARD syndrome, collectively known as neuro-cardio-facial-cutaneous syndromes. The high prevalence of somatic mutations within the human *RAS* genes (*KRAS*, *NRAS*, and *HRAS*) and within human *BRAF* in adult solid tumor epithelial cancers and melanoma, respectively, suggests that children bearing germline mutations in these genes would be pre-disposed to premature malignancy of epithelial origin or melanoma. In fact, these tumor types are uncommon in children with neuro-cardio-facial-cutaneous syndromes, and the most common tumor types reported are myeloid, myogenic, and neural tumors. Children with Costello syndrome and Noonan syndrome have the highest incidence of malignancies, with a cumulative incidence of cancer by age 20 years being 15% and 4%, respectively (Kratz *et al.*, 2011). The most common tumor types observed in Costello and Noonan syndromes are rhabdomyosarcoma and neuroblastoma. Bladder cancer of epithelial origin is also observed in adolescents with Costello syndrome. In addition to solid tumors, myeloproliferative disorder (MPD) is observed in children with Noonan syndrome. In the majority of cases, the MPD is transient and benign in nature; however, in some children, it progresses to full-blown juvenile myelomonocytic leukemia. Animal models including knock-in of *HRas*^{G12V}, which demonstrates development of squamous papillomas, and knock-ins of *KRas*^{G12D}, *Ptpn11*^{D61G}, or *Ptpn11*^{D61Y}, which demonstrate development of MPD, will be presented to demonstrate their utility in delineating molecular mechanisms underlying malignancies in children bearing these mutations.

Growth Hormone Signaling

Jessica Schwartz

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Growth Hormone (GH) is a major regulator of statural growth and metabolism. Changes in gene expression and in their regulatory transcription factors often underlie the physiological responses to GH. Recent insights in understanding GH signaling indicate that the interaction of GH with GH receptors on target cells initiates multiple signaling cascades that culminate in changes in transcription factors in the nucleus. Through analysis of profiles of GH-regulated genes, we find that in addition to well-recognized Stat5-mediated signaling, GH utilizes Ras/MAPK-mediated signaling to regulate the phosphorylation and function of C/EBP-CREB family transcription factors, which in turn modulate multiple genes in response to GH. Among these, GH-induced Ras/MAPK signaling mediates the phosphorylation of C/EBP beta, which contributes to the recruitment of the co-activator p300 in stimulating these genes. Thus, multiple signaling pathways are involved in mediating the ability of GH to regulate genes associated with its diverse physiological responses.

Noonan syndrome-associated Raf1 mutants with increased or decreased kinase activity differentially activate Erk and cause distinct syndromic phenotypes

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Noonan syndrome (NS) is one of several “RASopathies” caused by mutations in different components of the RAS-RAF-MEK-ERK MAPK pathway. Germ line mutations in *RAF1* (encoding the serine-threonine kinase RAF1) account for ~3-5% of NS. Unlike other NS alleles, *RAF1* mutations that confer increased kinase activity are highly associated with HCM. Surprisingly, other NS-associated *RAF1* mutations show normal or decreased kinase activity. To explore the pathogenesis of NS-associated *RAF1* mutations, we generated “knock-in” mice that express kinase-activating (L613V) and impaired (D486N) *RAF1* mutants, respectively. Like NS patients, L613V/+ mice had short stature, craniofacial dysmorphism and hematologic abnormalities. Valvuloseptal development was normal, but L613V/+ mice exhibited eccentric cardiac hypertrophy and aberrant cardiac fetal gene expression, and decompensated following pressure overload. D486N heterozygotes and, surprisingly, homozygotes, were obtained at the expected Mendelian ratio. Male D486N/+ mice did not show NS phenotypes, whereas D486N/+ females had mildly decreased body size. D486N/D486N mice showed cardiac hypertrophy, but not dilatation, and one third had severe growth defects. Agonist-evoked MEK/ERK activation was enhanced in multiple cell types from either Raf1^{L613V} or Raf1^{D486N} mutants, although L613V had a much stronger effect. Moreover, in response to agonist stimulation, both types of Raf1 mutant formed more heterodimers with Braf than did wild-type Raf1, and shRNA and mutational analysis indicate that heterodimers are required for mutant action. Hence, NS-associated kinase-active and impaired *RAF1* alleles act as gain-of-function mutants on the RAS-RAF-MEK-ERK pathway by promoting increased heterodimerization with BRAF. Finally, post-natal MEK inhibition normalized all NS defects in L613V/+ mice. We conclude that different NS genes have intrinsically distinct pathological effects, that enhanced MEK-ERK activity is critical for HCM and other RAF1-mutant NS phenotypes, and suggest a mutation-specific approach to RASopathy therapy.

Rapamycin reverses hypertrophic cardiomyopathy in a mouse model of LEOPARD syndrome-associated *PTPN11* mutation

Talita M. Marin*, Kimberly Keith*, Benjamin Davies, David Connor, Prajna Guha, Xue Wu, Clare Mahone, Michael Bauer, Sotirios Banakos, Roderick Bronson, Kleber Franchini, Benjamin G. Neel and Maria I. Kontaridis

LEOPARD syndrome (LS) is an autosomal dominant "RASopathy" disorder that manifests with congenital heart disease. Nearly all cases of LS are caused by catalytically inactivating mutations in the protein tyrosine phosphatase (PTP), non-receptor type II (*PTPN11*) gene that encodes the SH2 domain-containing PTP-2 (SHP2). RASopathies typically affect components of the RAS/MAPK pathway, yet it remains unclear how *PTPN11* mutations alter cellular signaling to produce LS phenotypes. We therefore generated knockin mice harboring the *Ptpn11* mutation Y279C, one of the most common LS alleles. *Ptpn11*^{Y279C/+} (LS/+) mice recapitulated the human disorder, with short stature, craniofacial dysmorphism, and morphologic, histologic, echocardiographic, and molecular evidence of hypertrophic cardiomyopathy (HCM). Heart and/or cardiomyocyte lysates from LS/+ mice showed enhanced binding of Shp2 to Irs1, decreased Shp2 catalytic activity, and abrogated agonist-evoked Erk/Mapk signaling. LS/+ mice also exhibited increased basal and agonist-induced Akt and mTOR activity. The cardiac defects in LS/+ mice were completely reversed by treatment with rapamycin, an inhibitor of mTOR. Our results demonstrate that LS mutations have dominant-negative effects in vivo, identify enhanced mTOR activity as critical for causing LS-associated HCM, and suggest that TOR inhibitors be considered for treatment of HCM in LS patients.

Mechanisms underlying the cognitive deficits in animal models of rasopathies

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Our laboratory has been studying the molecular and cellular mechanisms responsible for learning deficits in Rasopathies, a class of genetic disorders that alter Ras signaling mechanisms, and that result in a broad range of somatic, neurologic and psychiatric phenotypes. Although our work started with studies of neurofibromatosis type I (NF1), we have also studied several other rasopathies. For example, recently we uncovered molecular and cellular mechanisms responsible for the learning deficits associated with Noonan syndrome (NS), a genetic disorder with an incidence of 1 in ~2,500. As with NF1 patients, a significant percentage of NS patients show cognitive deficits, such as learning disabilities and mental retardation. Mutations in the *PTPN11* gene, which encodes the non-receptor protein tyrosine phosphatase SHP-2, account for ~50% of NS cases. Just as NF1, NS-associated SHP-2 mutants result in increases in Ras-ERK signaling, which is critically involved in learning and memory. However, unlike NF1, little is known about the role of SHP-2 in learning and memory and synaptic plasticity. Our laboratory recently found that similar to NF1 mouse mutants, mouse lines expressing two common NS-associated gain-of-function mutations show deficits in a hippocampus-dependent spatial learning task, as well as abnormalities in hippocampal long-term potentiation (LTP). To determine whether abnormal SHP-2 signaling in the adult brain is responsible for the behavioral deficits of these mutants, we overexpressed a NS-associated mutant SHP-2^{D61G} in the dorsal hippocampus of adult mice using viral vectors (rAAV). SHP-2^{D61G} overexpression resulted in hyperactivation of ERK signaling, deficits of both hippocampal LTP and spatial learning. As with NF1, a pharmacological manipulation that reduces ERK activation reversed the LTP and learning deficits in rAAV transfected mice, indicating that increased ERK signaling underlies the deficits in LTP and learning associated with NS. These results may not only lead to the development of a potential treatment for learning deficits in NS, they are also giving us insights into how disruptions in Ras signaling could result in learning and memory impairments. These insights will be critical for the development of general treatments for rasopathies.

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Induced Pluripotent Stem Cells

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The RASopathies comprise several phenotypically related genetic conditions arising from missense mutations in genes encoding proteins with roles in the RAS/mitogen-activated protein kinase (MAPK) pathway. Despite being defined genetically, much remains to be learned about the pathogenesis of disease, particularly with respect to understanding the specificity of how mutations in particular RAS/MAPK genes produce distinct phenotypes. While techniques for generating animal models such as in fruit flies, zebrafish and mice are available and being used to that end, there are potential advantages to studying diseases in human cells. A limitation in the field has been the relative inaccessibility of certain cell types from affected individuals and/or the inability to maintain such cells in long-term tissue culture.

Since the seminal paper from the Yamanaka group in 2007, the ability to generate induced pluripotent stem cells (iPSCs) from terminally differentiated human cells such as skin fibroblasts has enabled disease modeling with human cells. To that end and in collaboration with several investigators who are part of the RASopathy community, the Gelb lab has developed iPSC lines from several individuals with defined RAS/MAPK mutations. All lines were developed using the original Yamanaka approach with the four factors (c-MYC, OCT4, SOX2 and KLF4) in separate viruses. The resulting iPSCs were thoroughly characterized including demonstration of pluripotency *in vitro* and *in vivo*.

To date, work with the RASopathy iPSC cells has focused on two aspects of the clinical phenotype: hypertrophic cardiomyopathy and perturbed myeloid development. For this meeting, published and unpublished findings from these studies, showing that these phenotypes can be recapitulated *in vitro*, will be reviewed.

A *Drosophila* Approach to Ras Pathway Disease

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The Ras pathway is involved in a broad palate of diseases. Typically, it acts in conjunction with other pathways; this cross-talk occurs at multiple levels: signaling, cell, tissue, and organism. Failure to account for these complexities has led to limited success in therapeutics that target Ras pathway activity. We have utilized *Drosophila* to develop whole animal models designed to account for aspects of this complexity. I will discuss how our efforts highlight emergent properties as Ras signaling combines with other pathways in disease states such as cancers of the thyroid, breast, lung, and colon. We have developed these complex models both to explore emergent properties and as a whole animal screen for therapeutic compounds that attack multiple targets. Our drug surveys emphasize the importance of accounting for these multiple properties: drugs that attack Ras pathway alone work poorly in more complex models. Recently, we have collaborated with the Gelb laboratory to explore their model of Rasopathies including Noonan Syndrome. I will discuss our evidence that whole animal screening of even single mutation disease can benefit from therapeutics that address multiple targets.

Lovastatin, a cholesterol-lowering medication, improves cognitive deficits in children with Neurofibromatosis type I: Phase I study results

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Background: Cognitive deficits are the most important long term co-morbidity in NFI patients. In a mouse model of neurofibromatosis type I (NFI), lovastatin improved cognitive deficits and executive functioning.

Methods: Using a standard phase I study design, we examined the safety and biological impact of lovastatin as a treatment for the neurocognitive deficits in a group of 24 children 10-17 years old with diagnosis of NFI. A subset of patients underwent a 12-hour pharmacokinetics analyses.

Results: Lovastatin plasma concentration was varied between participants as area under the curve (AUC) profiles ranged from 2.61 ng*hr/mL to 14.05 ng*hr/mL. Change in LDL cholesterol levels during the study ranged from +10.5% to -44.6%. Medication was safe in all patients with minimal side effects, and the relation of cholesterol subtypes and other metabolic measurements stayed in the normal limits. Significant improvements ($p < .05$) were found several cognitive variables that deal with processing speed, verbal memory, nonverbal memory, and visuospatial skills (on the TEA-Ch, CVLT, WRAML2, and JLO, respectively). Additionally, change in LDL cholesterol was used in regression analyses to explain variance seen in the attention (on TEA-Ch) and memory (on CVLT) results.

Conclusions: Lovastatin was well tolerated and no safety concerns arose during the three-month period of our study. Despite small number of patients and variability in pharmacokinetics in the individuals, metabolism of cholesterol correlated with neuropsychological assessment. While the data is encouraging, further studies of drug efficacy on neuropsychological functioning are necessary. A phase 2 study is currently ongoing to further assess these observations.

Use of a genetically engineered murine model to identify novel experimental therapeutics for plexiform neurofibromas.

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Interactions between tumorigenic cells and their surrounding microenvironment are critical for tumor progression. Germline mutations in the *NF1* tumor suppressor gene cause neurofibromatosis type 1 (NF1), a common genetic disorder characterized by complex tumors called neurofibromas. Genetic studies indicate that biallelic loss of *Nf1* is required in the tumorigenic cell of origin in the embryonic Schwann cell lineage. However, in a genetically engineered murine model that is both genotypically and phenotypically similar to plexiform neurofibromas in NF1 patients, we have found that a multi-receptor tyrosine kinase inhibitor (imatinib mesylate) that targets c-kit, PDGF, and c-abl reduces plexiform neurofibromas. In addition in a proof of concept patient and in a pilot phase 2 trial, a number of human tumors stabilized or were reduced as well. Ongoing studies in the laboratory supported by a UOI mechanism are designed to evaluate whether other drugs that have a different spectrum of activity have comparable or greater efficacy as single agents or in combination with imatinib. Of the 10 drugs screened to date 2 drugs passed both short term and long term treatment objectives that included, reducing the size of the tumors by 50% and reducing the absolute number of tumors by 25% or more. Work is underway to move these drugs forward into phase 1-2 clinical trials.

Sleep disorders in Costello syndrome.

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Objective: to study sleep patterns and respiratory activity during sleep in a group of subjects with Costello syndrome (CS).

Methods: Fifteen consecutive patients, 7 males and 8 females, aged 3 to 29 years, affected by CS, were studied. The diagnosis was posed on the basis of established clinical criteria, and confirmed molecularly. All patients underwent clinical, neurological, otolaryngoiatric and radiologic evaluation. Sleep EEG was studied by means of full night, laboratory-based video-polysomnography, performed overnight, during hospitalisation. Sleep EEG was quantified by means of power spectral analysis.

Results: Polysomnography showed that 10 patients presented a relevant number of respiratory events of obstructive type during sleep. The apnea-hypopnea index (AHI) ranged from 0 to 21.2 events per hour (mean index = 8.5 ± 6.9 events/hour). Five patients, 3 males and 2 females, age range 9-31 months, presented, during sleep, repeated, stereotyped movements of the tongue producing a sucking-like or licking-like movement, mostly during NREM sleep. All patients exhibited increased EEG power in 12-15 Hz activity band compared to age-matched controls.

Conclusions: Costello patients have a complex abnormality of the sleep pattern, which includes sleep fragmentation, parasomnias, sleep disordered breathing and peculiar EEG features.

NSEuroNet – a progress report about the European database

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The European network on Noonan syndrome and related disorders (NSEuroNet) is a EU-funded collaborative project that started its activities in 2010. The project is coordinated by Marco Tartaglia and aims at improving the knowledge about RASopathies at the levels of basic research as well as of practical clinical care. We provide a progress report on the RASopathy mutation and phenotype database that is a core part of the NSEuroNet project.

This registry is aimed at providing locus-specific mutation databases for the RASopathy genes in combination with standardized phenotype data including long-term follow-up. The ultimate goals are to clarify genotype-phenotype correlations, to detect rare manifestations (e.g. cancer) and possible associated risk genotypes, to improve counseling and individual clinical care, and to develop a platform from which clinical trials could be carried out. Compliance and interactivity with other mutation database projects (Human Variome Project, LOVD, MutaBASE) is sought. The current status of the NSEuroNet activities will be described and put up for discussion.

YOUNG INVESTIGATOR FINALISTS

Characterisation of Costello syndrome mutations in sperm and implications for genetic counselling

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Costello and Noonan syndromes are part of a small class of congenital syndromes called 'paternal age-effect' (PAE) disorders. Other members of this class include Apert and Crouzon syndromes (associated with *FGFR2* mutations), achondroplasia, thanatophoric dysplasia (*FGFR3* mutations) and MEN2a/b (*RET* mutations). In the vast majority of cases, these PAE disorders are caused by spontaneous point mutations and exhibit the collective properties of (1) very high apparent rates of germline base substitution (~500-1000-fold over background), (2) exclusive paternal origin, and (3) an increased average age of the unaffected father from whom the mutation originates (PAE). As somatic events, the same gain-of-function mutations have been associated with various human cancers.

These unusual features have led us to study some of these ultra-rare mutations (such as the Apert 755C>G *FGFR2* and thanatophoric dysplasia 1949A>G *FGFR3* substitutions) directly in human sperm where we showed that they accumulate over time in the sperm of most men. We have also identified somatic *FGFR3* (1949A>G) and *HRAS* (181C>A and 182A>G, encoding Q61K and Q61R) mutations in spermatocytic seminoma, a rare type of testicular tumour. The combined evidence suggests that these PAE mutations, although occurring rarely, provide a selective growth advantage to the mutant spermatogonial stem cells, resulting in their clonal expansion and a relative enrichment in sperm over time, accounting for the PAE. To date, all examples of PAE mutations result in the activation of the growth factor receptor-RAS signalling pathway, which is a key determinant of spermatogonial stem cell proliferation and renewal.

To further characterise the mechanisms associated with PAE, we have recently studied most of the Costello syndrome-associated mutations located at the G12 codon of *HRAS* directly in human sperm, using massively parallel sequencing. These mutations were detected at significant levels (ranging from <1:1,000,000 to 1:8,000 across 92 samples) in the sperm of most men and shown to increase exponentially with the age of the donor, accounting for the PAE observed for Costello syndrome. Our results indicate that the G12S (34G>A) substitution is by far the most common and the most abundant *HRAS* mutation in sperm (average ~1:45,000), followed by the G12D (35G>A) (average ~1:125,000), while the G12C (34G>T) and the G12V (35G>T) mutations were observed in a smaller number of samples and at lower levels (average ~1:300,000-1:400,000, respectively). Unexpectedly, we also identified many events of tandem substitutions in sperm. In the case of the strongly activating G12V change, tandem mutations (35_36GC>TT and 35_36GC>TA, which have been associated with several Costello cases) are more common than single substitutions (35G>T, reported only in one Costello patient), suggesting an unusual mechanism of mutagenesis taking place within the testis. The relative abundance of *HRAS* mutations we observed in sperm is consistent with the documented prevalence of Costello alleles and suggests that the strongly activating alleles (such as G12V and G12D), although causing severe phenotypes, do not simply lead to foetal lethality.

These data are also useful for genetic counselling of families presenting with *de novo* cases of Costello syndrome (and likely extends to all other RASopathies) as the mechanisms associated with PAE mutations in sperm predicts a very low risk of recurrence, which can be usually estimated to be <1:1,000.

Germline K-Ras^{V14I} mutation causes Noonan Syndrome and fatal myeloproliferative disorder

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Noonan syndrome (NS) is an autosomal dominant disorder characterized by short stature, distinctive facial dysmorphism, cardiac defects and increased risk of leukemia. Approximately 50% of NS cases are associated with germline mutations in *PTPN11*. Other known NS genes include *SOS1*, *RAF1*, *BRAF*, *K-RAS*, *N-RAS* and *SHOC2*. Although *K-RAS* mutations are found in a low percentage of clinically diagnosed patients (<3%), they usually correlate with the appearance of more severe symptoms. These mutations result in milder activation of the K-RAS protein and are different from the somatic mutations found in human tumors. We have generated a K-Ras knock-in mouse model that carries a germline K-Ras^{V14I} mutation, one of the alleles identified in NS patients. K-Ras^{+V14I} mice are viable indicating that K-Ras^{V14I} expression is compatible with embryo and postnatal development. However, homozygous K-Ras^{V14I/V14I} animals display high perinatal lethality due to cardio-respiratory problems. Both heterozygous and homozygous mutant mice display some of the features observed in patients with NS, including small size, craniofacial abnormalities and cardiomegaly. Furthermore, expression of the K-Ras^{V14I} allele is associated with short life span due to a fatal myeloproliferative disorder characterized by leukocytosis, splenomegaly and myeloid cell expansion in the spleen. Flow cytometry analysis also revealed an increase of the hematopoietic stem cell pool in the bone marrow of these animals. These mice should help to better understand the molecular and cellular bases of the defects associated with NS and to evaluate preventive as well as therapeutic strategies.

Identification of novel genes critical to survival of malignant peripheral nerve sheath tumor cells via medium throughput screening using lenti-viral shRNA

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Malignant peripheral nerve sheath tumor (MPNST) is a soft tissue sarcoma that is highly invasive and eventually lethal. About 50% of MPNSTs have mutation and/or loss of the *NF1* gene, while the rest are spontaneous. Many MPNSTs also show mutations in *p53* and/or homozygous deletion of *CDKN2*. In an effort to identify novel genes that play key roles in MPNST tumorigenesis, we performed a genome wide micro-array analysis using human neurofibroma and MPNST (Miller et al., 2009). This analysis identified 130 genes that are up-regulated >3 fold in MPNSTs as compared to normal human Schwann cells. Over expression of these genes in tumors as compared to normal cells suggested that some might be critical for survival of MPNST cells. This hypothesis was directly tested by studying effects on survival of MPNST cells *in vitro* post down regulation of the 130 genes using lenti-viral short hairpin (sh) RNA. For each shRNA experiment a shnon-targeting lenti-virus was used as a negative control and shSOX9, a construct previously shown to kill MPNST cells *in vitro*, was used as a positive control for an effect on cell survival. Screening was performed sequentially allowing for selection of genes with significant effects on survival. In screen I, T265 MPNST cells were exposed to each shRNA virus. In screen II, shRNAs were tested for effects on adenocarcinoma cells (A549) vs T265 cells. These two rounds of screens identified 8/130 genes specific for survival of T265 cells. Screen III was performed on four different MPNST cell lines, including T265 (*NF1*^{-/-}), 8814 (*NF1*^{-/-}), S462TY (*NF1*^{-/-}) and SSTS26T (sporadic MPNST wild-type for *NF1*), to identify shRNA's with similar or different effects on cell survival in the presence or absence of wild-type *NF1* gene. No significant difference in survival was observed for NF null MPNST cells vs sporadic MPNST cell line for any gene. This approach facilitates a streamlined pathway from genome wide gene expression analysis to identification of 8 novel genes critical to survival of cancer cells *in vitro* and potential therapeutic targets toward a cure for MPNSTs.

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POSTER ABSTRACTS

Epidemiological features of Costello Syndrome and Cardio-facio-cutaneous Syndrome: findings from the first nationwide survey

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Costello syndrome and cardio-facio-cutaneous (CFC) syndrome are a group of genetic disorders which result from dysregulation of the RAS/MAPK cascade. Germline mutations in HRAS are causative for Costello syndrome and those in KRAS, BRAF and MAP2K1/2 (MEK1/2) cause CFC syndrome. Since our discovery of HRAS mutations in Costello syndrome (2005) and KRAS/BRAF mutations in CFC syndrome (2006), approximately 200 patients of each syndrome have been reported. However, clinico-epidemiological features of these disorders remain to be elucidated. In order to assess the prevalence, natural history, prognosis and tumor incidence, we conducted a nationwide prevalence study of patients with Costello syndrome and CFC syndrome in Japan. The study consisted of two-stage questionnaire surveys, which were distributed to a total of 1127 departments, including randomly selected pediatric and genetic departments at hospitals and institutions for severely-retarded children. The first survey inquired about the number of mutation-positive patients as well as clinically suspected patients with Costello syndrome and CFC syndrome. The second asked for detailed clinicoepidemiological information of each patient reported. The response rate of the first-stage survey was 76% (856/1127). Sixty-three patients with HRAS-positive Costello syndrome and 64 patients with CFC syndrome with mutations with KRAS, BRAF or MAP2K1/2 were reported, including 9 new patients with Costello syndrome and 8 new patients with CFC syndrome analyzed in the current study. The total numbers of patients with Costello syndrome and CFC syndrome in Japan were estimated as 105 (95% confidence interval, 84 to 127) and 160 (95% confidence interval, 88 to 232), respectively. The secondary survey revealed age distribution and the frequency of heart defects, mental retardation and tumor association. Sixteen adult patients aged 18-32 years were reported. Ten of the sixteen patients can walk alone and one of them had developed recurrent bladder papillomata. The prevalence of Costello syndrome and CFC syndrome were estimated as 1 in 1,230,000 population and 1 in 790,000 population, respectively. This is the first epidemiological study of both disorders. Identification of patients older than 32 years of age and follow up of the reported patients in the current study will be important to estimate the precise prevalence and natural history of these disorders.

MOUSE MODELS OF COSTELLO SYNDROME

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Costello Syndrome (CS) is a rare, complex, developmental disorder characterized by a number of features including— failure to thrive, characteristic facies, delay in intellectual development, hypertrophic cardiomyopathy, arrhythmia, and predisposition to both benign and malignant tumors. Past studies identifying gain-of-function mutations in the *HRAS* gene as the basis of human CS, and strong conservation with the mouse ortholog *Hras1*, have led to the development of a Gly12Val (G12V) mouse model of CS. To extend animal modeling studies to include various allelic forms of the disease, we are developing five additional mouse models of CS. Using a recombineering-based “knock-in” approach, we have completed construction of five vectors, each containing a *loxP*-flanked, neomycin resistance (*Neo^r*) selection cassette in intron 1 of *Hras1* and site-directed mutations encoding each of the following five pathogenic alleles — G12A encoded by GCA, G12A encoded by GCC, G12S encoded by AGC, G12V encoded by GTA and G12V encoded by GTT. Four of the five strains have been completed creating animal models for the G12A^{GCA}, G12A^{GCC}, G12V^{GTA} and G12V^{GTT} forms of the disease. Suppression of each CS-causative allele by the oppositely transcribed *Neo^r* transcript can be relieved by breeding affected mice to inner cell mass- or germline-specific “deleter” *Cre* lines such as *Hprt-Cre* or *Alpl-Cre*, respectively. Abnormalities have been observed for all mutant alleles but appear to be most pronounced in the two G12V lines where there is a higher degree of lethality than in the two G12A alleles. For all alleles, mutant mice display a tremor and appear less robust than their wild type littermates. Craniofacial abnormalities are also apparent in all mutant lines. Preliminary phenotyping of a small number of mutants suggests cardiac defects (including mitral valve abnormalities and abnormal ECG patterns), enlarged regions of the brain (including the pineal gland), possible neoplasia and skin abnormalities. Each strain will be deposited into The Jackson Laboratory Repository. Together, these five models hold the potential for uncovering allelic and codon preference influences on development and neoplasia in CS, identifying CS modifying genes, and dissecting tissue-specific facets of CS using spatially-controlled *Cre* driver lines.

Costello Syndrome

Hras1

Conditional mutagenesis

Piebaldism with Multiple Café-au-lait Macules and Intertriginous Freckling: Evidence for a Common Pathway between KIT and SPRED1

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A 5 year old boy presented with a congenital depigmented patch of the forehead, as well as acquired white forelock, depigmentation of the medial eyebrows, and depigmented patches of the body. Additionally, he had started to develop multiple café-au-lait macules (CALMs) and freckling of the axillae and inguinal folds. His past history was significant for mild asthma, chronic sinusitis, surgical excision of a thyroglossal duct cyst, and umbilical hernia repair. There was no developmental delay or hearing loss. Given concerns by other providers for tuberous sclerosis, neurofibromatosis type I (NFI), and Waardenburg syndrome, the patient had a prior work-up consisting of a normal brain MRI, echocardiogram, audiogram, and ophthalmology exam. His family history was significant for similar skin findings in his father, paternal half-brother, paternal grandmother, and paternal great-grandfather.

On examination, there was a diamond-shaped, depigmented patch on the midline forehead with adjacent white forelock and poliosis of the medial eyebrows. On the midline chest and bilateral calves were depigmented patches. Scattered on the entire body were numerous CALMs, twelve of which were >1 cm in size. There were subtle freckles present in the bilateral axillae and inguinal folds. There were no neurofibromas, iris heterochromia, or dystopia canthorum. There was relative macrocephaly; head circumference was 50th percentile, while height and weight were 25th percentile. *KIT* gene testing was performed, and a novel missense mutation was detected in the intracellular tyrosine kinase domain.

Piebaldism is a disorder of melanocyte development resulting in leukoderma (white skin) and poliosis (white hair), characteristically a white forelock.¹ It is caused by an autosomal dominant mutation in the *KIT* proto-oncogene.² The *KIT* protein product is a receptor tyrosine kinase that activates several intracellular signaling pathways. Legius syndrome is characterized by multiple CALMs and axillary or inguinal freckling, without other features of NFI such as neurofibromas, Lisch nodules, and central nervous system tumors.³ Autosomal dominant loss-of-function mutations in *SPRED1*, resulting in induction of the Ras/MAP kinase pathway, is the cause of Legius syndrome.⁴ Phosphorylation of *SPRED1* by kinases such as *KIT* is required for activation and efficient suppression of the Ras/MAP kinase pathway.^{4,5}

There have been five patients reported to have congenital depigmentation consistent with piebaldism, as well as multiple CALMs and intertriginous freckling.^{6,9} The prior published reports attribute the cutaneous findings to the co-existence of piebaldism with NFI. We believe that these cases are better classified as a variant of piebaldism, where loss of *SPRED1* function due to inadequate phosphorylation by *KIT* is the cause for the CALMs and freckling seen in some patients. To date, *SPRED1* mutations and Legius syndrome have not been associated with neurofibromas and other tumors, suggesting that piebaldism patients with multiple CALMs and axillary or inguinal freckling may not need the rigorous monitoring that NFI patients require.

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Sinking our teeth into Costello syndrome: Ras signaling regulates enamel deposition in humans and mice.

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Ras/MAPK signaling is critical in animal development, and receptor-tyrosine kinase signaling, which activates Ras signaling, is known to play an important role in tooth development. Our previous work has shown that increasing Ras/MAPK signaling by inactivating Sprouty genes adversely affects tooth morphogenesis. Here, we directly examined the effects of activating Ras/MAPK signaling in both humans and mice. Costello Syndrome (CS) is caused by a heterozygous de novo germline mutation in HRAS that results in a constitutively active Ras protein. We examined a cohort of CS patients at the 2009 Costello Syndrome International Conference and identified a number of craniofacial and dental anomalies. The most striking finding was that a large majority of patients presented with a pronounced enamel defect. Micro computed tomography of exfoliated primary teeth from CS patients showed a significant decrease in enamel thickness compared to controls. We next examined the CS mouse model and found that the mice also had an enamel defect. Further inspection revealed disorganization of the ameloblasts in the mouse incisor. We are currently studying cell proliferation and polarity of the ameloblasts in the mutant mice incisors and using an ameloblast-like cell line. Together, our studies point to a role for Ras signaling in regulation of cell polarity and deposition of mineralized matrices.

The PI3K Catalytic Subunits, p110a and p110d, Serve Redundant Functions in Activating *PTPN11*-Induced Hematopoietic Progenitor Hypersensitivity to GM-CSF.

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Noonan syndrome (NS) is a common (1 in 1500 to 2500 live births) autosomal-dominant disorder caused by somatic gain-of-function mutations in *PTPN11* in approximately 40 - 50% of patients. *PTPN11* encodes Shp2, a non-receptor protein tyrosine phosphatase, that has been demonstrated repeatedly to play a positive role in growth factor signaling to Ras in a phosphatase-dependent manner. Although the anomalies observed in NS are diverse, several of the complications can be attributed to the increased function or number of macrophages or monocyte-derived cells including transient and remitting myeloproliferative disorder and, rarely, development of full-blown juvenile myelomonocytic leukemia (JMML). JMML is characterized clinically by overproduction of myelomonocytic cells and by the *in vitro* phenotype of hematopoietic progenitor hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF). We have previously demonstrated that Phosphoinositol-3-Kinase (PI3K) signaling is hyperactivated in the presence of gain-of-function mutant Shp2 and that it contributes to activating *PTPN11*-induced GM-CSF hypersensitivity, which can be reduced pharmacologically with the pan-PI3K inhibitor, LY294002. To study the contribution of Class IA PI3K, we genetically disrupted *Pik3r1*, which encodes regulatory subunits p85 α , p55 α , and p50 α , and found significant, but incomplete, reduction of GM-CSF-stimulated hyperproliferation in gain-of-function Shp2 E76K-expressing cells. The relative contribution of the different catalytic subunits to mutant Shp2-induced PI3K hyperactivation and GM-CSF hypersensitivity is not known, though we found that in the absence of the *Pik3r1*-encoded regulatory subunits, p110d expression is significantly higher in cells expressing gain-of-function mutant Shp2 E76K compared to WT Shp2. To investigate this question further, we measured GM-CSF-stimulated proliferation of WT Shp2- and Shp2 E76K-transduced hematopoietic progenitors in the presence and absence of GDC-0941, a PI3K inhibitor with high specificity for p110a and p110d, and found a dose-dependent decrease in GM-CSF-stimulated proliferation. To distinguish the role of p110a and p110d in Shp2 E76K-induced hyperproliferation, we performed genetic studies using mice bearing a conditional knockout of *Pik3ca* (encoding p110a) and a kinase-dead knock-in of *Pik3cd* (p110d D910A), respectively. Following GM-CSF stimulation, we found that genetic disruption of p110a or expression of kinase dead p110d D910A partially normalized Shp2 E76K-mediated Akt hyper-phosphorylation; however, GM-CSF-stimulated proliferation was unchanged. These findings indicate that p110a and p110d functionally perform redundant tasks in Shp2 E76K-expressing cells. We are currently crossing these genetically modified animals to further define the functional contribution of p110a and p110d to mutant Shp2-induced Akt hyperactivation and hypersensitivity to GM-CSF.

A novel *HRAS* substitution (c.266C>G; p.S89C) resulting in decreased downstream signaling reveals a new dimension of RAS pathway dysregulation in human development

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Costello syndrome was delineated based on its distinctive phenotype including coarse facial features, severe failure to thrive, intellectual disability, cardiac abnormalities and a high malignancy risk. Costello syndrome is caused by germline mutations in *HRAS* encoding a small GTPase which cycles between an inactive, GDP-bound and an active, GTP-bound state. Mutations in >90% affect Gly¹² or Gly¹³ and are associated with a relatively homogeneous Costello syndrome phenotype. The same amino acid substitutions occur as somatic changes in malignant tumors and result in constitutive *HRAS* activation and increased RAF-MEK-ERK and PI3K-AKT signal flow. A few less common germline missense mutations affecting other *HRAS* codons were reported in patients with a distinctive, usually attenuated or mild, Costello syndrome (p.T58I, p.K117R and p.A146T/V), or in individuals with a predominant muscular phenotype (p.Q22K and p.E63K) [1]. These changes were also suspected or proven to enhance *HRAS*-dependent signaling.

Here we report a novel heterozygous *HRAS* alteration, c.266C>G (p.S89C), in a female (Pt. 1) presenting with severe fetal hydrops and pleural effusion, followed by a more benign postnatal course including a renal cyst and strabismus. A brother (Pt. 2) with the same mutation and fetal polyhydramnios showed a Dandy-Walker malformation and his postnatal course was complicated by severe feeding difficulties. Their apparently asymptomatic father is heterozygous for the same change; however, we did not detect this alteration in 488 control alleles. In functional studies of COS-7 cells ectopically expressing various *HRAS* mutants, the binding domains of the *HRAS* effector proteins RAF1, PI3K, and RALGEF were used to specifically pull-down active *HRAS*. Upon growth factor-stimulation, co-precipitation of *HRAS*^{S89C} with any tested effector was decreased compared to wild-type *HRAS*, indicating a reduced growth factor-dependent activation of *HRAS*^{S89C}. Accordingly, we detected slightly diminished MEK, ERK and AKT phosphorylation in cells overexpressing *HRAS*^{S89C}. Thus, p.S89C appears to reduce downstream signaling, a novel consequence of disease-associated *HRAS* mutations.

The *C. elegans* RAS homologue *let-60* is essential for vulval induction and activating gain-of-function mutations, such as p.G13E, result in multivulval organisms. Notably, expression of *let-60* with the amino acid change p.S89F results in a vulvaless phenotype, thereby supporting our functional data [2].

The decreased downstream signaling effect of *HRAS*^{S89C} clearly differs from those for typical CS-associated *HRAS* mutations. Given the Patient 1's benign postnatal course and presence of this change in her asymptomatic father, its harmful consequences may be time limited, with the late fetal stage being most sensitive. While the physical findings in both patients are not typical for Costello syndrome, they are consistent with a dysregulation of the RAS/MAPK pathway. Together, these data illustrate the wide functional and phenotypic variability of germline *HRAS* mutations.

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Neurofibromatosis Networking – Building the Tools to Advance Research Discoveries to the Clinic

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Objective: Neurofibromatosis (NF) encompasses a group of rare disorders affecting 100,000 Americans. The clinical course of NF is unpredictable: it can lead to deafness, blindness, amputation or disfigurement. The genes for the two major forms, NF1 and NF2, were identified in the early 1990’s, and the molecular signaling pathways are well understood. The primary funders of US NF research are the Congressionally Directed Medical Research Program for Neurofibromatosis Research (\$10M-\$25M/year) and NIH (\$11-\$15M/year). Major tools developed in the last 10 years include transgenic mouse models representing individual NF manifestations, and a Phase II Clinical Trials Consortium. In 2006, the Children’s Tumor Foundation (CTF) examined the NF research landscape looking for ‘funding gaps’ that if filled could rapidly advance NF discoveries to the clinic. The goal was to implement recommendations within 5 years.

Methods: We first mapped ‘bench to bedside’ the dollar amounts of NF grants issued by NIH and CDMRP 1996-2005, then convened an expert strategic planning workshop. We concluded that the NF community had a reasonable understanding of NF candidate drug targets; good preclinical drug screening tools; and a Phase II clinical trials consortium; but that areas in need of funding were: i) Coordinated preclinical drug screening; ii) pilot clinical trials for initial advancement of promising compounds; iii) NF clinic network; iv) patient registry; and v) tissue bank to utilize for identifying new biomarkers.

Results: CTF has now launched initiatives in all five 2006 strategic recommendation areas. These include: a NF Preclinical Consortium and Drug Discovery Initiative to test candidate drugs in multiple NF tumor models; a Clinical Trial Awards program for proof of concept studies; an NF Clinic Network of 44 US clinics; and we are in the process of developing both a Patient Registry and Tissue Biobank.

Conclusions: In early 2011 CTF reviewed these initiatives for the next five years’ strategic planning 2012-2016. Lessons learned have included vagaries of managing multisite legal agreements - particularly when collaborating with a commercial entity; setting realistic goals with measurable milestones; managing donor and constituent expectations. On the positive side, we have a significant increase in industry’s interest in NF as a marketable condition. Preclinical testing, clinical trials and clinical care are becoming progressively linked and the NF community can now offer industry the opportunity to engage in NF research at any point from pilot preclinical studies to large scale clinical trials.

Induced pluripotent stem cell-derived cardiomyocytes as a model to study cardiac defects in Noonan syndrome and related disorders

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Mutations in *PTPN11* and *BRAF*, genes involved in the RAS/MAPK pathway, are implicated in a variety of clinical disorders, such as Noonan syndrome (NS), LEOPARD syndrome (LS), and cardio-facial-cutaneous syndrome (CFCS), members of a family of disorders termed the “RASopathies”. These disorders are characterized by skeletal and neurological defects, as well as a high prevalence of cardiovascular abnormalities. Hypertrophic cardiomyopathy (HCM) is observed in 90% of patients with *PTPN11* mutations causing LS and 40% of patients with CFCS, yet *PTPN11* mutations causing NS are negatively associated with HCM. In order to further elucidate the molecular mechanisms of RASopathy-associated HCM, we have generated human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes as representative models of these syndromes. We have previously demonstrated that hiPSCs derived from patients with LS exhibit altered RAS/MAPK signaling and their derived cardiomyocytes display a molecular phenotype consistent with the cardiac hypertrophy observed clinically. We have now introduced lentiviral selection cassettes driven by the cardiomyocyte-specific gene *α-myosin heavy chain (αMHC)* (gift from Mark Mercola), in order to purify cardiomyocytes from a heterogeneous population of hiPSC-derived cells. Additionally, the use of bacterial artificial chromosomes (BACs) containing selection markers driven by the ventricular cardiomyocyte specific gene *myosin light chain 2v (MLC2V)*, will allow specific isolation and characterization of ventricular cells, and thus more thorough investigation of the mechanism underlying RASopathy-associated HCM. We hypothesize that purified hiPSC-derived cardiomyocytes from patients harboring LS *PTPN11* mutations and CFCS *BRAF* mutations will display increased cellular area and altered signaling pathway activation compared to hiPSC-derived cardiomyocytes from patients harboring NS *PTPN11* mutations and control cardiomyocytes. Using a directed cardiac differentiation protocol, we have obtained differentiated beating embryoid bodies (EBs) in all samples. After selection with the *αMHC* selection cassette, counting cardiac troponin T+ (cTNT) cells revealed an enrichment of up to 90% cardiomyocytes. ImageJ analysis of cTNT+ cells revealed a similar average cellular area between CFC-BRAF and LS-PTPN11 samples, double in size compared to the average cellular area in the NS-PTPN11 sample. Our data indicate that the phenotype of iPSC-derived cardiomyocytes from patients with LS, CFCS, and NS correlates with the clinically observed cardiac phenotype of their respective disorders. Using purified cardiomyocytes from these lines, we will be able to study the molecular mechanisms underlying the cardiovascular manifestations characteristic of their corresponding disorders, with the goal of further understanding the role of RAS/MAPK signaling in normal and abnormal cardiac function.

Pro-hypertrophic signaling induced by the Shp2 mutation Q510E in mice

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Rationale: The identification of mutations in *PTPN11* (encoding the protein tyrosine phosphatase Shp2) in families with congenital heart disease has facilitated mechanistic studies of various cardiovascular defects. However, the roles of normal and mutant Shp2 in the developing heart are still poorly understood. We focused our studies on the Q510E mutation in Shp2, which is associated with a particularly severe form of hypertrophic cardiomyopathy in patients. It is still under debate whether patients with this particular mutation rather fall into the Noonan Syndrome or into the LEOPARD Syndrome category since clinical differentiation between these syndromes can be very challenging, in particular in infants. To unravel the underlying disease mechanism of this aggressive mutation, we tested the biochemical characteristics of the Q510E-Shp2 protein and investigated the downstream signaling events triggered by this mutation in a mouse model.

Results: Using purified mouse Q510E-Shp2 protein for biochemical assays, we found that the Q510E mutation nearly completely abolished phosphatase activity of the Shp2 protein. In cultured rat cardiomyocytes expressing both Q510E-Shp2 and normal Shp2, Q510E-Shp2 downregulated signaling through the ERK/MAPK pathway in response to growth factor stimulation. This indicates that Q510E acts as a dominant-negative loss-of-function mutation, thus closely resembling other loss-of-function mutations found in LEOPARD Syndrome families.

To test the effects of Q510E-Shp2 on the whole organ level, we generated transgenic mice with cardiomyocyte-specific expression of this mutation starting either before or after birth. Mice with Q510E-Shp2 expression starting before birth developed early-onset hypertrophic cardiomyopathy. In neonatal mice, we found increased cardiomyocyte sizes, heart-to-body weight ratios, septum thickness, and cardiomyocyte disarray. In young adult mice, this was also accompanied by cardiac fibrosis. Echocardiographically, these hearts showed reduced contractile function and increased wall thicknesses, thus closely resembling the human disease characteristics associated with this mutation. In tissue samples taken from Q510E-Shp2-expressing heart muscle, signaling through the pro-hypertrophic Akt/mTOR pathway was significantly increased. Importantly, inhibition of this pathway with the mTOR inhibitor rapamycin rescued the Q510E-Shp2-induced hypertrophy. In contrast, in a second mouse model in which expression of Q510E-Shp2 started after birth, mice did not develop hypertrophic cardiomyopathy.

Conclusions: 1. The Q510E-Shp2 mutation displays similar biochemical characteristics and induces hypertrophic cardiomyopathy through the Akt/mTOR pathway as recently found with other loss-of-function mutations in Shp2 associated with LEOPARD Syndrome. Therefore, our data support the classification of Q510E-Shp2 as a LEOPARD Syndrome mutation. 2. Cardiomyocyte-specific expression of Q510E-Shp2 is sufficient to induce hypertrophic cardiomyopathy in mice, indicating that the cardiomyocytes themselves are responsible for driving the phenotype. However, a supporting role for cardiac fibroblasts in the disease process can not be excluded at this time. 3. The time window of Q510E-Shp2 expression is critical for triggering disease. Only expression of Q510E-Shp2 during heart development, but not starting after birth, induces hypertrophic cardiomyopathy in mice. 4. However, rapamycin treatment started after birth can still reverse the phenotype, which may have important therapeutic implications for patients carrying the Q510E-Shp2 mutation.

How Loss of Neurofibromin in Oligodendrocytes Affects the Brain

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Neurofibromatosis type 1 patients are predisposed to central nervous system (CNS) phenotypes including enlarged brains, delayed acquisition of motor skills, brain tumors, and cognitive deficits. Imaging and pathologic analysis suggest that changes in white matter myelination may underlie both the enlargement of white matter tracts that contributes to megacephaly, and/or hyper-intense signals visualized on MRI. To study the role(s) of *Nf1* and HRas in oligodendrocytes, we examined the optic nerve and corpus callosum, myelinated fiber tracts. We studied *Nf1* heterozygous mice, tamoxifen-induced *Nf1* loss in mature oligodendrocytes (Plp-CreERT), and a new transgenic model in which the CNPase promoter drives expression of *HRasG12V*. Activated HRas and loss of *Nf1* within oligo-lineage cells (*PLPCre; Nf1^{f1+}*; & *PLPCre; Nf1^{f1/f1}*) resulted in optic nerve enlargement. The corpus callosum of *CNP-HRasG12V* mice was also enlarged. Electron microscopy analysis revealed 3 phenotypes within the enlarged optic nerves. 1) When *Nf1* was lost or HRas was activated within oligodendrocytes, the myelin was decompacted due to splitting at the intraperiod lines. The transgenic *Nf1^{+/-}* mice, in which *Nf1* loss is not restricted to oligo-lineage cells, displayed lesser myelin decompaction, and these mice did not have significantly enlarged optic nerves. 2) Enlarged axons accompanied the decompacted myelin within all models. 3) All *Nf1* and Ras mouse models also showed an expansion of the perivascular astrocytic endfeet surrounding the vasculature. These phenotypes were also found within the corpus callosum. Thus, myelin and vascular phenotypes are not limited to a single myelinated fiber tract. These studies reveal a cell autonomous role for the *Nf1*/Ras pathway in the regulation of myelin compaction, and a non-cell autonomous role in the regulation of astrocytic endfeet surrounding brain capillaries.

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Perinatal or Adult *Nf1* Inactivation using Tamoxifen-inducible *PlpCre* Each Cause Neurofibroma Formation

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OBJECTIVES

Neurofibromas are tumors initiated by biallelic mutation of the *Nf1* tumor suppressor gene in the Schwann cell lineage. One idea within the field suggests that *Nf1* loss must occur within progenitor cells present within a critical window during Schwann cell development in order for neurofibromas to form. To test this hypothesis and to examine whether myelinating Schwann cells can serve as a neurofibroma cell of origin, *Nf1* loss was induced at perinatal or adult timepoints using a tamoxifen-inducible *Plp-CreERT* driver.

RESULTS

Perinatal loss of *Nf1* resulted in small neurofibromas late in life, while adult loss caused large neurofibromas and morbidity beginning 4 months after onset of *Nf1* loss. *PLP-CreERT* recombination (EGFP⁺ cells) occurred in: satellite cells, S100β⁺ myelinating Schwann cells, and p75⁺ cells. *Plp-CreERT* nerves and neurofibromas contained cells with Remak bundle disruption; however, no recombination within GFAP⁺ non-myelinating Schwann cells was identified. Extramedullary lympho-hematopoietic expansion that contained EGFP⁺/Sca-1⁺ stromal cells amongst EGFP⁻ negative lympho-hematopoietic cells was also observed.

CONCLUSIONS/SIGNIFICANCE

Neurofibroma formation is not restricted to loss of *Nf1* in embryonic life, but can be triggered by *Nf1* loss throughout life. Although all neurofibroma models and human samples have Remak bundle disruption (leading to the assumption that *Nf1* loss within the non-myelinating Schwann cell may be vital for tumor formation), there was no EGFP⁺ recombination within GFAP⁺ non-myelinating Schwann cells – eliminating the GFAP⁺ non-myelinating Schwann cell as the cell of origin for neurofibroma formation.

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Hematopoietic differentiation abnormalities in Noonan syndrome and Noonan/JMML iPSC cells

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BACKGROUND: Noonan syndrome (NS) is a genetic developmental disorder caused by deregulation of the RAS/MAPK pathway. Germ-line mutations in *PTPN11*, which encodes SHP-2, a key component of the RAS/MAPK pathway, cause 50% of NS, while somatic mutations in this gene account for 35% of juvenile myelomonocytic leukemia (JMML). Children with NS and specific *PTPN11* mutations are at increased risk for developing JMML, inferring that certain SHP-2 mutants result in abnormal differentiation and cell maturation in hematopoietic lineages. The molecular mechanisms resulting from SHP-2 dysregulation that lead to these abnormalities remain largely unexplored.

AIM: To elucidate signaling pathway alterations in myeloid progenitors in NS and NS/JMML using human induced pluripotent stem cells (hiPSC) derived from patients with those disorders.

DESIGN/METHODS: We established two human iPSC lines as control samples and eight iPSC lines with germ-line mutations in specific residues of *PTPN11*: Y63C and E76D in NS samples and D61H and G503C in NS/JMML samples. We differentiated these iPSCs into hematopoietic lineages using specific cytokines. Hematopoietic populations (surface markers: CD33, CD14, CD11b, CD71, CD235a, and CD41) in these samples were determined with flow cytometry. Proliferation and apoptosis were determined with Ki67 and Annexin V staining, respectively. To check clonogenic capacity, cells were plated on methylcellulose with specific cytokines to obtain CFU-GMs and CFU-Es. We assessed two clinical criteria used routinely for definitively diagnosing JMML: hypersensitivity to GM-CSF and absence of BCR-ABL fusion gene by FISH. Using RT-PCR and western blotting, we analyzed the levels of *STAT5*, *BCL-XL* and a panel of specific miRNAs (miR181, miR128a, miR20a, miR17, miR106 and miR223) in both the mixed hematopoietic population and CD33+ myeloid progenitors.

RESULTS: We observed an increase of myeloid progenitors (45%), monocytes (18%), erythrocytes (37%) and megakaryocytes (15%) in NS/JMML compared to controls (15%, 8%, 25% and 8% respectively). In addition, we observed an increase in the size and the total number of colonies in NS/JMML (600 vs 200 total colonies). The NS-JMML lines showed hypersensitivity to GM-CSF, responding at 0.1 ng/ml to which controls were not responsive. BCR-ABL fusion was absent. Apoptosis tended to be decreased in NS/JMML. While proliferation rate was marginally increased in mixed hematopoietic NS/JMML cells compared to controls, it was increased approximately 8 fold in CD33+ NS/JMML cells. These changes in proliferation and apoptosis in CD33+ NS/JMML cells correlated with increased expression of *BCL-XL* and *STAT5* compared to control CD33+ myeloid cells. After screening of miRNAs associated with differentiation of hematopoietic cells using RT-PCR, we observed that the expression level of miR223, a specific regulator of granulocyte/monocyte precursors, was increased 500 fold in the mixed population and 20 fold in the myeloid population with mutations of *PTPN11* while the expression levels of the other miRNAs assessed were comparable to controls.

CONCLUSIONS: This study provides the first model of leukemia using hematopoietic cells differentiated from human iPSCs. Moreover, these studies provide new insights about *PTPN11*-driven JMML, revealing up-regulation of *STAT5* and miR223. These findings provide potential novel molecular targets for treating JMML, which remains a lethal disorder. Our future work will be directed at determining the upstream regulators and downstream effects of increased *STAT5* and miR223. We are also attempting to use the NS/JMML iPSC-derived hematopoietic progenitors to develop a transplant mouse model of JMML.

HRAS mutants identified in Costello syndrome patients can induce cellular senescence: possible implications for the pathogenesis of Costello syndrome

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Costello syndrome (CS) is a congenital disease that is characterized by a distinctive facial appearance, failure to thrive, mental retardation and cardiomyopathy. In 2005, we identified that heterozygous germline mutations in HRAS caused CS. Several studies have shown that CS-associated HRAS mutations are clustered in codons 12 and 13, and mutations in other codons have also been identified. However, a comprehensive comparison of the substitutions identified in patients with CS has not been conducted. In the current study, we identified four mutations (p.G12S, p.G12A, p.G12C and p.G12D) in 21 patients and analyzed the associated clinical manifestations of CS in these individuals. To examine functional differences among the identified mutations, we characterized a total of nine HRAS mutants, including seven distinct substitutions in codons 12 and 13, p.K117R and p.A146T. The p.A146T mutant demonstrated the weakest Raf-binding activity, and the p.K117R and p.A146T mutants had weaker effects on downstream JNK signaling than did codon 12 or 13 mutants. We demonstrated that these mutant HRAS proteins induced senescence when overexpressed in human fibroblasts. Oncogene induced senescence is a cellular reaction that controls cell proliferation and is driven by oncogenic mutation, and it has been considered one of the tumor suppression mechanisms in vivo. Our findings suggest that the HRAS mutations identified in CS are sufficient to cause oncogene induced senescence and that cellular senescence might therefore contribute to the pathogenesis of CS.

High-throughput drug screening to identify novel small molecules to rescue Noonan syndrome phenotypes in *Drosophila*

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BACKGROUND: Noonan syndrome (NS) is a pleiomorphic developmental disorder, for which increased RAS pathway signaling is the primary cause. Gain-of-function mutations in *PTPN11*, encoding SHP-2, are the major cause. While powerful Ras signaling inhibitors are in development for cancer, such drugs may not be useful for long-term use in children with NS. To find drugs that restore normal Ras signaling in NS, we are using a transgenic *Drosophila* model with the D61G mutation in corkscrew mutation (*csw*^{D61G}), the fly orthologue of *PTPN11*.

AIMS: We aimed to establish conditions for high-throughput drug screening with the *csw*^{D61G} fly and to screen a limited panel of biologically relevant drugs to demonstrate proof of principle.

DESIGN/METHODS: To establish conditions of pupal lethality, which is optimal for high-throughput chemical screening, we crossed the *UAS-csw*^{D61G} allele with several different *Gal4* drivers (*daughterless*, *actin*, *tubulin* and *patched*) and cultured at temperatures ranging from 18-29°C to alter transgene expression levels. After establishing optimal driver and culture conditions, we performed a screen employing a panel of biologically relevant drugs at two concentrations (50 and 100 µM).

RESULTS/CONCLUSIONS: Most drivers were embryonic lethal, precluding their use, but *patched-Gal4* exhibited 100% lethality at 23 °C, semi-viability at 21 °C, and full viability at 18 °C. Using 23 °C as the “barely lethal” condition, we screened 14 drugs/chemicals acting on RAS, NOTCH, mTOR and PI3 kinase signaling. The mTOR and PI3 kinase inhibitors, rapamycin and wortmannin, respectively, failed to rescue lethality. The remaining 12 compounds achieved rescue at varying extents. Sunitinib, which is a multiple kinase inhibitor including RTK, had maximal rescue of 60% at 50 µM. Sorafenib, a multiple RTK and RAF inhibitor, rescued more consistently nearly 40% of pupae, so will be used for future chemical library screening. Of interest, we also observed rescue by inhibitors of JNK (SP5600125), AKT (10_DEBC), Rac (NSC23766), and Notch/Presinilin (DAPT). We are now initiating a screen of 640 FDA-approved compounds using a 96-well plate format with robotic dispensing of the embryos.

Hematologic abnormalities associated with patients with cardio-facio-cutaneous syndrome

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Cardio-facio-cutaneous (CFC) syndrome is a multiple congenital anomaly/mental retardation syndrome characterized by a distinctive facial appearance, ectodermal abnormalities and heart defects. Clinically, it overlaps with both Noonan syndrome and Costello syndrome. Mutations in *KRAS*, *BRAF* and *MAP2K1/2* (*MEK1/2*) have been identified in patients with CFC syndrome. By age 20, the cumulative incidence of cancer was approximately 4% for Noonan syndrome and 15% for Costello syndrome (Christian P. Kratz. *AJMD*, 2011). For Costello syndrome, tumor-screening protocols have been proposed. In contrast, little attention has been paid to the development of tumors in patients with CFC syndrome. We have previously reported three CFC patients with *BRAF* mutations who developed hematologic malignancies: two patients with acute lymphoblastic leukemia, 1 with non-Hodgkin lymphoma. A patient with a *MAP2K1* mutation, who developed hepatoblastoma, has also been reported. Here we report additional patient with CFC syndrome who developed juvenile myelomonocytic leukemia (JMML)-like myeloproliferative disorder. He was delivered by caesarian section at 32 weeks' gestation due to non-immune hydrops fetalis. He had curly hair, low-set ears, atrial septal defect, hepatosplenomegaly, pulmonary arteriovenous fistula and [portosystemic shunt](#). At one month age, the patient showed marked monocytosis and decrease in platelet count. The diagnosis of JMML was made on bone marrow cell culture studies. Hematological abnormalities resolved spontaneously at three month of age. Sequencing of genomic DNA from the patient showed a heterozygous p.T241P (c.721A>C) mutation. This is the first report that a patient with CFC syndrome developed JMML-like myeloproliferative disorder. The clinical features of JMML-like disorder in the patient are similar with those of JMML-like disorder/Noonan syndrome. Germline mutations in *BRAF* possibly contribute to leukemogenesis and JMML-like abnormalities with spontaneous regression in early infantile period. These results suggest the importance of molecular diagnosis and careful observation in children with CFC syndrome.

The eye in rasopathies- phenotypic variation and possible role of modifiers

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The role of the Ras/MAPK signaling in eye and vision development is being increasingly studied and shown to be important in, in-vitro studies, animal models including zebra fish and mouse models and in individuals having rasopathies.

We studied 58 individuals including 28 with Cardiofaciocutaneous (CFC), 16 with Costello syndrome and 14 with Noonan syndrome during Berkeley Ras/MAPK symposium. Additionally we had collected visual exam reports from individuals not in this study. Both similarities and differences in the ocular presentation were noted in all three syndromes. The most serious visually disabling problem not amenable to treatment currently was optic nerve hypoplasia. However, not all individuals had optic nerve involvement. About 30% of CFC, 10-20% of Costello children and 10-20% of Noonan individuals have optic nerve involvement.

Most individuals had either or frequently both 1).ocular alignment problems resulting in strabismus– exotropia (an eye or eyes turning outward), esotropia (inward crossing of the eyes), hypertropia (one eye being higher than the other) and 2). refractive errors including myopia (near-sightedness), hyperopia (far-sightedness) and astigmatism. These issues commonly resulted in problems with depth perception, abnormal head posturing and amblyopia (diminished visual acuity due to unequal eye use). Functional studies showed that most individuals had reasonably good vision in one or both eyes, but lacked depth perception. Some other issues included nystagmus (rhythmic shaking of the eyes) and ptosis (droopy eye-lids).

Even though all of these individuals have disruption of the Ras/MAPK pathway, there is a wide range of phenotype variation. A good example is Costello syndrome, caused by missense mutations in the gene *HRAS*, with the Gly12Ser substitution accounting for >80% of all individuals with a known mutation with only few individuals having optic nerve hypoplasia. This suggests the role for modifier genes or environmental factors predisposing one individual to develop optic nerve hypoplasia or alternatively protecting another from developing it. If we are able to understand these factors better it would help not only the individuals with CFC, Costello and Noonan but may also help in developing therapeutic options for optic nerve hypoplasia in general population.

We propose to do genotype-phenotype correlation studies in affected individuals and also study ocular phenotype in animal models to better understand the pathogenic mechanisms contributing to these eye development disorders.

A role for synaptic plasticity in the cognitive deficits of Costello syndrome mice

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Costello syndrome is a rare RASopathy caused by activating mutations in the HRAS gene. Cognitive impairments and behavioral problems are common amongst children with Costello syndrome. To understand the underlying mechanisms of these deficits, mouse models have been developed in which the endogenous HRAS gene has been mutated to express constitutively active *Hras*^{G12V}, similar to patients. *Hras*^{G12V}-mice showed spatial learning deficits in the Morris Water Maze, in the absence of motor performance problems or gross brain pathology. We found a marked increase in activity dependent MAPK-phosphorylation in hippocampal tissue. Since Ras-MAPK signaling has been implicated in synaptic plasticity, we subjected *Hras*^{G12V} brains to hippocampal field recordings. Input/output properties of *Hras*^{G12V} brains were normal, suggestive of normal anatomy and connectivity. However, synaptic plasticity as measured by the ability to undergo long-term potentiation (LTP) was affected. These differences were also present in mice that specifically expressed the *Hras*^{G12V} allele in neurons. Taken together, our data suggests a role for impaired synaptic functioning in the mechanisms of cognitive deficits of Costello syndrome. Ras-inhibitors are currently being tested to see whether the cognitive phenotype of *Hras*^{G12V} mice is reversible.

Molecular genetic analysis of the protein tyrosine phosphatase Shp2 (PTPNI 1) in the mouse telencephalon

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Shp2 (PTPNI 1) is an intracellular protein tyrosine phosphatase that is critical for mediating cell signaling in response to growth factors. Germline mutations in PTPNI 1 have been identified Noonan and LEOPARD syndrome patients. Patients with either syndrome exhibit several congenital malformations including a variety of cardiovascular and skeletal defects. In addition to these defects, there is also an increased incidence of learning disabilities and cognitive impairment observed in both syndromes. The specific brain abnormalities that lead to these neurocognitive defects remain unknown. The goal of our study is to utilize mouse genetics to understand the role of Shp2 (PTPNI 1) in the telencephalon, which is the region of the brain most associated with higher neural functions like cognition and emotion. To do this, we have generated a conditional deletion of *Shp2* and a conditional misexpression of the Q79R Noonan Syndrome mutation in transgenic mice restricted to the ventral telencephalon and oligodendrocyte lineage with *Olig2*^{cre/+} mice. Our results suggest that Shp2 is crucial for progenitor cell development in the embryonic telencephalon and in the generation of myelinating oligodendrocytes in telencephalic structures of the brain. Current experiments are focused on identifying regional roles for Shp2 (PTPNI 1) in the development of cell types originating from the telencephalon and determining the cellular origins of the neurocognitive defects observed in these patients.

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