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Molecular and Clinical Analysis of *RAF1* in Noonan Syndrome and Related Disorders: Dephosphorylation of Serine 259 as the Essential Mechanism for Mutant Activation

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ABSTRACT: Noonan syndrome (NS) and related disorders are autosomal dominant disorders characterized by heart defects, facial dysmorphism, ectodermal abnormalities, and mental retardation. The dysregulation of the RAS/MAPK pathway appears to be a common molecular pathogenesis of these disorders: mutations in *PTPN11*, *KRAS*, and *SOS1* have been identified in patients with NS, those in *KRAS*, *BRAF*, *MAP2K1*, and *MAP2K2* in patients with CFC syndrome, and those in *HRAS* mutations in Costello syndrome patients. Recently, mutations in *RAF1* have been also identified in patients with NS and two patients with LEOPARD (multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness) syndrome. In the current study, we identified eight *RAF1* mutations in 18 of 119 patients with NS and related conditions without mutations in known genes. We summarized clinical manifestations in patients with *RAF1* mutations as well as those in NS patients with *PTPN11*, *SOS1*, or *KRAS* mutations previously reported. Hypertrophic cardiomyopathy and short stature were found to be more frequently observed in patients with *RAF1* mutations. Mutations in RAF1 were clustered in the conserved region 2 (CR2) domain, which carries an inhibitory phosphorylation site (serine at position 259; S259). Functional studies revealed that the RAF1 mutants located in the CR2 domain resulted in the decreased phosphorylation of S259, and that mutant RAF1 then dissociated from 14-3-3, leading to a partial ERK activation. Our results suggest that the dephosphorylation of S259 is the primary pathogenic mechanism in the activation of RAF1 mutants located in the CR2 domain as well as of downstream ERK.
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KEY WORDS: RAS; MAPK; RAF1; Noonan syndrome; PTPN11; hypertrophic cardiomyopathy

Introduction

Noonan syndrome (NS; MIM# 163950) is an autosomal dominant developmental disorder characterized by facial dysmorphism, including hypertelorism, low-set ears, ptosis, short stature, skeletal abnormalities, and heart defects [Allanson et al., 1985; Mendez and Opitz, 1985]. Frequently observed features in NS patients are pulmonary stenosis (PS), hypertrophic cardiomyopathy, chest deformities, a webbed/short neck, mental

Additional Supporting Information may be found in the online version of this article.

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retardation, genitourinary defects including cryptorchidism in males, and bleeding diathesis due to factor XI deficiency. The incidence of this syndrome is estimated to be 1 in 1,000–2,500 live births. LEOPARD (multiple *l*entigines, *e*lectrocardiographic conduction abnormalities, *o*cular hypertelorism, *p*ulmonary stenosis, *a*bnormal genitalia, *r*etardation of growth, and sensorineural *d*eafness) syndrome (MIM# 151100) is known to be a NS-related disorder [Digilio et al., 2002]. The features of NS overlap with those of Costello syndrome and cardio-facio-cutaneous (CFC) syndrome. Patients with Costello syndrome (MIM# 218040) show distinctive facial features, mental retardation, high birth weight, neonatal feeding problems, curly hair, nasal papillomata, deep skin creases at palms and soles, and hypertrophic cardiomyopathy [Hennekam, 2003]. CFC syndrome (MIM# 115150) is characterized by distinctive facial features, mental retardation, heart defects (PS, atrial septal defect [ASD], and hypertrophic cardiomyopathy), and ectodermal abnormalities such as sparse, friable hair, hyperkeratotic skin lesions, and a generalized ichthyosis-like condition [Reynolds et al., 1986].

The molecular pathogenesis of these syndromes has been investigated. Tartaglia et al. [2001] have identified missense mutations in *PTPN11*, a gene encoding protein tyrosine phosphatase (PTP) SHP-2, in 45% of clinically diagnosed NS patients. Specific mutations in *PTPN11* has been identified in patients with LEOPARD syndrome [Digilio et al., 2002]. In 2005, we identified *HRAS* germline mutations in patients with Costello syndrome [Aoki et al., 2005]. Mutations in *KRAS*, *BRAF*, and *MAP2K1/2* have been identified in those with CFC syndrome [Niihori et al., 2006; Rodriguez-Viciana et al., 2006]. Mutations in *KRAS* and *SOS1* have also been identified in patients with NS [Roberts et al., 2007; Schubbert et al., 2006; Tartaglia et al., 2007]. Mutations in *NF1* and *SPRED1* have been identified in patients with neurofibromatosis type I (MIM# 162200) [Brems et al., 2007]. These findings suggest that dysregulation of the RAS/RAF/MEK/ERK pathway causes NS and related disorders, and thus it has been suggested that these syndromes be comprehensively termed the RAS/MAPK syndromes [Aoki et al., 2008] or the neuro-cardio-facial-cutaneous syndrome [Bentires-Alj et al., 2006].

In 2007, gain-of-function mutations in *RAF1* were identified in 3–17% of patients with NS and two patients with LEOPARD syndrome [Pandit et al., 2007; Razzaque et al., 2007]. RAF1 is a member of the RAF serine–threonine kinase family and transmits the upstream RAS signaling to downstream MEK and ERK. RAF1, ARAF, and BRAF share three conserved regions, CR1, CR2, and CR3 [Mercer and Pritchard, 2003]. Mutations in *BRAF* identified in patients with CFC syndrome are clustered in CR1 and CR3 domains [Aoki et al., 2008]. In contrast, reported *RAF1* mutations in NS and LEOPARD syndrome were located in the CR2 domain and some mutations were located in CR3 domain. These mutants had enhanced RAF1 kinase activities and most mutations, but not all, showed enhanced phosphorylation of ERK1/2 [Pandit et al., 2007; Razzaque et al., 2007]. Pandit et al. [2007] suggested that RAF1 mutations might interfere with RAF1 phosphorylation at serine 259 as well as with 14-3-3 interaction, and reported that p.P261S did not bind to 14-3-3. However, the mechanisms of RAF1 activation in mutants remain unexplained.

In the present study, we analyzed the *RAF1* gene in 119 patients with NS and related phenotypes without mutations in *PTPN11*, *HRAS*, *KRAS*, *BRAF*, *MAP2K1/2*, and *SOS1*. Detailed clinical manifestations in our new patients with *RAF1* mutations were evaluated, and those in patients with *RAF1 KRAS*, *PTPN11*, and *SOS1* mutations previously reported by us and others were examined. Furthermore, we explored the molecular mechanisms by which RAF1 mutants are activated.

Materials and Methods

Patients

One hundred nineteen patients with NS or related phenotypes were recruited. The primary diagnoses made by clinical dysmorphologists and general pediatricians were as follows: 44 patients with NS, 46 patients with CFC syndrome, 25 patients with Costello syndrome, and 4 patients with atypical phenotypes. No mutations in *PTPN11*, *HRAS*, *KRAS*, *BRAF*, *MAP2K1*, *MAP2K2*, or *SOS1* were identified in these patients. Control DNA was obtained from 105 healthy Japanese individuals. Control DNA from 105 healthy Caucasian individuals was purchased from Coriell Cell Repositories (Camden, NJ). This study was approved by the Ethics Committee of Tohoku University School of Medicine. We obtained informed consent from all subjects involved in the study and specific consent for photographs from six patients.

Mutation Analysis in *RAF1*

Genomic DNA was isolated from the peripheral blood leukocytes of the patients. Each exon with flanking intronic sequences in *RAF1* was amplified with primers based on GenBank sequences (Supp. Table S1; GenBank accession no. NC_000003.10). The M13 reverse or forward sequence was added to the 5′ end of the polymerase chain reaction (PCR) primers for use as a sequencing primer. PCR was performed in 30 μl of a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10% (v/v) DMSO, 24 pmol of each primer, 100 ng genomic DNA, and 1.5 units of Taq DNA polymerase. The reaction conditions consisted of 35 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec, and extension at 72°C for 40 sec. The products were gel-purified and sequenced on an ABI PRISM 310 or 3130 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Determination of the RAF1 phosphorylation status

The expression construct, including a *RAF1* cDNA (pUSEamp-*RAF1*), was purchased from Millipore (Billerica, MA). A Myc-tag was introduced at the 5′ terminus of the cDNA by PCR and the PCR product was subcloned into pCR4-TOPO (Invitrogen, Carlsbad, CA). The entire cDNA was verified by sequencing. A single-base substitution resulting in p.H103Q, p.R191I, p.S257L, p.S259F, p.P261A, p.N262K, or p.S427G was introduced using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All mutant constructs were verified by sequencing. The Myc-tagged wild-type *RAF1* cDNA and mutant cDNAs were digested with *Eco*RI and *Eco*RV and subcloned into the *Eco*RI–*Eco*RV site of the pUSEamp-*RAF1*.

COS7 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in DMEM containing 10% fetal calf serum (FCS), 50 U/ml penicillin, and 50 μg/ml streptomycin. COS7 cells were seeded at 1×10^5 cells per 6-cm dish, and 24 hr later, 2.0 μg of pUSE vectors encoding one of the wild-type (WT) or mutant RAF1 cDNAs were transfected using 8 μl of PLUS Reagent and 12 μl of Lipofectamine Reagent (Invitrogen). After 3 hr, the medium was replaced to complete medium. After 48-hr culture, cells were scraped and collected by centrifugation after two washes with phosphate-buffered saline

(PBS). Lysates were prepared in 100-μl lysis buffer (10 mM Tris-HCl pH 8.0 and 1% SDS) and boiled for 3 min. The DNA was sheared with a syringe. The lysates were centrifuged at 14,000 × *g* for 15 min at 4°C and protein concentration was determined by Bradford assay. Thirty micrograms of protein was subjected to SDS-polyacrylamide gel electrophoresis (5–20% gradient gel) (ATTO, Tokyo, Japan), transferred to nitrocellulose membrane, and probed with anti-Myc antibody and phospho-specific RAF1 antibodies (Cell Signaling, Danvers, MA). All the membranes were visualized using a Western Lightning ECL-Plus Kit (Perkin-Elmer, Norwalk, CT). The following antibodies were used for Western blotting: anti-Myc (9E10, Santa Cruz Biotech, Santa Cruz, CA), antiphospho-c-Raf (S259) (Cell Signaling), antiphospho-c-Raf (S338) (Millipore), antiphospho-c-Raf (S289/296/301) (Cell Signaling), antiphospho-c-Raf (S621) (Millipore), and antineomycin phosphotransferase II (Millipore).

For immunoprecipitation, lysates were prepared in 1 ml of ice-cold RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1:100 protease inhibitor (Sigma, St. Louis, MO), 1:1000 phosphatase inhibitor (Sigma), and 1% Triton X) and incubated on ice for 15 min. Four hundred micrograms of protein was incubated with anti-Myc (9E10) antibody for 1 hr at 4°C. Immune complexes were collected by adding 50 μl of 50% protein G-Sepharose bead slurry (GE Healthcare, Milwaukee, WI) for 1 hr at 4°C, washed three times with RIPA buffer, and then boiled in 2 × SDS buffer. The samples were resolved in 5–20% gradient polyacrylamide gels, transferred to nitrocellulose membranes and probed with antiphospho-c-Raf (S259) and anti-Myc (9E10) antibodies.

Reporter Assay

NIH 3T3 cells (ATCC) were maintained in DMEM containing 10% newborn calf serum, 50 U/ml penicillin, and 50 μg/ml of streptomycin. One day prior to the transfection, the NIH 3T3 cells were plated in 12-well plates with a density of 1×10^5 cells per well. Cells were transiently transfected using Lipofectamine and PLUS Reagents with 700 ng of pFR-luc, 15 ng of pFA2-Elk1, 7 ng of phRLnull-luc, and 35 ng of WT or mutant expression constructs of *RAF1*. Eighteen hours after transfection, the cells were cultured in DMEM without serum for 24 hr. Cells were harvested in passive lysis buffer, and luciferase activity was assayed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Renilla luciferase expressed by phRLnull-luc was used to normalize the transfection efficiency. The experiments were performed in triplicate. Data are shown as mean ± SD. Statistical analysis was performed using Excel.

Binding of RAF1 with 14-3-3

An expression construct containing Myc- and Flag-tagged 14-3-3 ζ (pCMV6-14-3-3 ζ) was purchased from Origene (Rockville, MD). In order to remove the Myc-tag from the construct, the 3′ half of the cDNA and the Myc-tag were removed by digestion with *Eco*RV and the 3′ half of cDNA was filled using PCR. An S621A mutation, which impairs phosphorylation of S621 to bind 14-3-3, was introduced into pUSE RAF1 harboring WT, p.S257L, or p.N262K cDNA by a Quickchange Site-Directed Mutagenesis Kit. HEK293 cells (ATCC) were transfected with 2 μg RAF1 constructs and 2 μg pCMV6-14-3-3 ζ construct using Lipofectamine and PLUS Reagents. After 48 hr, cells were scraped and collected by centrifugation after two washes with PBS. Lysates were prepared as described above. The Myc-tagged RAF1 was immunoprecipitated with anti-Myc antibody (clone4A6, Millipore) for 1 hr at 4°C. Immune complexes were collected by adding 50 μl of 50% protein G-Sepharose bead slurry (GE Healthcare) for 1 hr at 4°C, washed three times with RIPA buffer, and then boiled in 2 × SDS buffer. The samples were resolved in 5–20% gradient polyacrylamide gels, transferred to nitrocellulose membranes, and probed with anti-FLAG M2 (Sigma) and anti-Myc antibodies. For immunoprecipitation of 14-3-3, anti-FLAG M2 antibody was used and immunoblotting was performed using anti-FLAG M2 and anti-c-Raf (Cell Signaling) antibodies.

Results

Mutation Analysis in Patients

We identified eight amino acid changes in 18 patients (Table 1). A C-to-T nucleotide change, resulting in an amino acid change p.S257L, was identified in 11 patients. Novel p.R191I (c.572G>T) and p.N262K (c.786T>A) were identified in one each patient. Previously reported mutations, including p.S259F (c.776C>T), p.P261A (c.781C>G), p.P261L (c.782C>T), p.S427G (c.1279A>G), and p.L613V (c.1837C>G), were identified in a single patient. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in Genbank NM_002880.3, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. The mutation p.S427G, which has been reported in a patient with therapy-related acute myeloid leukemia [Zebisch et al., 2006], was identified in one patient. None of the newly identified mutations were observed in the control DNA of 105 ethnically matched healthy subjects. Parental samples were obtained from six patients (NS86, 92, 209, 210, 222, and 258). The analysis showed that p.S257L, p.P261A, and p.N262K occurred de novo. p.S427G was also identified as well in his 32-year-old mother, who also exhibited a Noonan phenotype with distinctive facial appearance, sparse hair in infancy, and multiple lentigines. The p.H103Q (c.309C>G) was identified in patient NS86, in whom p.S257L was also identified. This amino acid change was identified in one of his parents without any clinical features, suggesting that this amino acid change was polymorphic.

Clinical Manifestations of Patients with *RAF1* Mutations

Initial diagnoses of patients with *RAF1* mutations were as follows: NS in 11 patients, CFC syndrome in 4 patients, and Costello in 3 patients (Supp. Table S2). Four patients who were first diagnosed as having CFC syndrome were reclassified as NS because of facial features and normal mental development after identification of *RAF1* mutations. Three patients were diagnosed as having Costello syndrome. One patient was rediagnosed as having NS (NS135) and the other patient died at 1 month (NS209). Detailed information on clinical manifestations of NS205 was not available.

Detailed clinical manifestations in 18 patients with *RAF1* mutations were evaluated (Table 2 and Fig. 1). Nine of 15 patients had prenatal abnormality, including cystic hygroma, polyhydramnions, and asphyxia. Most patients had characteristic craniofacial abnormalities frequently observed in NS: relative macrocephaly (94%), hypertelorism (93%), downslanting palpebral fissures (63%), epicanthal folds (86%), and low-set ears (93%). Mental retardation was observed in 6 of 11 (55%) patients. Short stature (73%), short neck (93%), and webbing of neck (81%) were also observed. As for cardiac abnormalities, hypertrophic cardiomyopathy was observed in 10 of 16 patients (63%), followed by pulmonic stenosis (47%),

Table 1. *RAF1* Mutations Identified in This Study*

Patient ID	Country of origin	Final diagnosis	Exon	Nucleotide change	Amino acid change	Domain	Genotype of father/mother
NS213	France	atypical NS	5	c. 572G>T	p.R191I[a]	CR1	NA
NS39	Japan	NS	7	c.770C>T	p.S257L	CR2	NA
NS86	France	NS	3, 7	c.309C>G	p.H103Q	CR1,	H103Q/WT
				c.770C>T	p. S257L	CR2	WT/WT
NS92	Germany	NS	7	c.770C>T	p.S257L	CR2	WT/WT
NS135	Japan	NS	7	c.770C>T	p.S257L	CR2	NA
NS146	Spain	NS	7	c.770C>T	p.S257L	CR2	NA
NS199	Japan	NS	7	c.770C>T	p.S257L	CR2	NA
NS200	France	NS	7	c.770C>T	p.S257L	CR2	NA
NS215	Japan	NS	7	c.770C>T	p.S257L	CR2	NA
NS227	Japan	NS	7	c.770C>T	p.S257L	CR2	NA
NS256	Japan	NS	7	c.770C>T	p.S257L	CR2	NA
NS258	Japan	NS	7	c.770C>T	p.S257L	CR2	WT/WT
NS279	Japan	NS	7	c.776C>T	p.S259F	CR2	NA
NS210	France	NS	7	c.781C>G	p.P261A	CR2	WT/WT
NS205	France	CS[b]	7	c.782C>T	p.P261L	CR2	NA
NS209	France	CS[c]	7	c.786T>A	p.N262K[a]	CR2	WT/WT
NS222	Japan	NS	12	c.1279A>G	p.S427G[d]	CR3	WT/p.S427G
NS285	Japan	NS	17	c.1837C>G	p.L613V	CR3	NA

NS, Noonan syndrome; CS, Costello syndrome; WT, wild type; CR, conserved region; NA, not available.
*GenBank RefSeq: NM_002880.3 Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.
[a]Novel mutation.
[b]Detailed clinical manifestations were not obtained.
[c]The patient died at 1 month.
[d]The mutation was previously identified in a patient with a therapy-related acute leukemia.

ASD (31%), arrhythmia (38%), and mitral valve anomaly (29%). Other observed clinical features were hyperelastic skin (58%), curly hair (47%), and cryptorchidism in males (50%). Coagulation defects were observed in two patients.

Four patients with *RAF1* mutations died before 5 years of age (Supp. Table S2). Patient NS39 were diagnosed as having cystic hygroma in the prenatal period and had suffered from neonatal hypertrophic cardiomyopathy. At 1 year of age, she contracted acute respiratory distress syndrome after having pneumonia and died of respiratory failure. Patient NS199 had been suspected to have achondroplasia because of short limbs. He was diagnosed as having NS at 3 years of age because of distinct facial features, growth failure, short stature, and hypertrophic cardiomyopathy. He had pneumonia without fever for a week and died suddenly at 5 years of age. Patient NS227 suffered from feeding difficulties, ectopic atrial tachycardia, as well as VSD and pulmonary hypertension. The patient died at 2 months of tachycardia (>200/min) and laryngeal edema.

Clinical manifestations in our patients with *RAF1* mutations were compared with those previously reported (Table 2). The high frequency of hypertrophic cardiomyopathy in our study (63%) was consistent with that observed in patients with *RAF1* mutations previously reported (77%). The frequency of ASD and that of mitral valve anomaly were similar to those of the previous studies. However, the frequency of PS in our study (47%) was higher than that previously reported (11%). Arrhythmia was less frequently observed in our patients with *RAF1* mutations (38 vs. 89%). The frequency of mental retardation (55%) was almost same as that of the previous studies (56%). Hyperelastic skin (58%) and coagulation defects (two cases) were also described in previously reported patients with *RAF1* mutations (24% and one case, respectively).

Phosphorylation State of Mutant RAF1 Proteins

RAF1 is a ubiquitously expressed RAF serine/threonine kinase, which regulates the RAS pathway. It has been shown that phosphorylation of serine, threonine, and tyrosine residues contributes to a conformational change of RAF1 protein and activation in growth factor stimulation [Mercer and Pritchard, 2003]. In the inactive state, phosphorylated S259 and S621 serve as binding sites for 14-3-3, leading to a closed confirmation [Dhillon et al., 2007]. Phosphorylation of S621 seems essential for RAF1 activation. In contrast, phosphorylation of serine 259 has been shown to have an inhibitory role in RAF1 activation. When cells are stimulated with growth factors, dephosphorylation of S259 by protein phosphatase 1 (PP1) and/or protein phosphatase 2A (PP2A) promotes the dissociation of 14-3-3 from RAF1, resulting in an activated conformation of RAF1 protein. For full activation, multiple residues, including S338, are phosphorylated and substrate of RAF1 enters the catalytic cleft in the CR3 kinase domain. Negative feedback from activated ERK results in the phosphorylation of S289, 296, and 301 [Dhillon et al., 2007].

To examine the phosphoryalation status of mutants observed in NS patients, we transfected constructs harboring WT *RAF1* cDNA and five mutants identified in NS patients. Immunoblotting was performed using four phospho-specific antibodies of RAF1 (Fig. 2A). We first analyzed the phosphorylation status of two phosphorylation sites, S259 and S621, using antibodies that recognize each site. Immunoblotting showed that phosphorylation of S259 was scarcely observed in cell lysates expressing p.S257L and p.N262K. In contrast, phosphorylation of S259 of p.H103Q, p.R191I, and p.S427G was similar to that in WT RAF1. To confirm this observation, immunoprecipitation was performed using an anti-Myc antibody, and phosphorylation levels at S259 were examined (Fig. 2B). Immunoprecipitated RAF1 mutants (p.S257L and p.N262K) were not phosphorylated at S259, confirming that these mutants had impaired phosphorylation of S259. The phosphorylation level of S621 in four mutants (p.H103Q, p.R191I, p.S257L, and p.N262K) was similar to that in WT (Fig. 2A), whereas that in cells expressing p.S427G was enhanced. Phosphorylation levels at S338 and S289/296/301 were similar to that in WT except for p.S427G (Fig. 2A).

Phosphorylation levels at S259, S289/296/301, S338, and S621 were shown to be enhanced in cells expressing p.S427G. The expression of p.S427G appeared enhanced and the band was

Table 2. Clinical Manifestations in *RAF1*-Positive Patients in This Study and Past Studies

	Present cohort (%)	NS with *RAF1* mutations (%)	LS with *RAF1* mutations (%)
Number of patients in total	17	35[a]	2
Perinatal abnormality			
Polyhydramnios	6/15 (40)	6/19 (32)	ND
Fetal macrosomia	5/11 (45)	6/20 (30)	ND
Growth and development			
Failure to thrive in infancy	10/12(83)	3	ND
Mental retardation	6/11 (55)	19/34 (56)	1
Outcome			
Died	4/17 (24)	2/11 (18)	ND
Craniofacial characteristics			
Relative macrocephaly	16/17 (94)	16/21 (76)	ND
Hypertelorism	14/15 (93)	20/21 (95)	2
Downslanting palpebral fissures	10/16 (63)	19/21 (90)	2
Ptosis	9/16 (56)	19/21 (90)	1
Epicanthal folds	12/14 (86)	12/21 (57)	1
Low-set ears	14/15 (93)	18/21 (86)	2
Skeletal characteristics			
Short stature	11/15 (73)	30/35 (86)	2
Short neck	14/15 (93)	21/31 (68)	2
Webbing of neck	13/16 (81)	25/30 (83)	2
Cardiac defects			
Hypertrophic cardiomyopathy	10/16 (63)	27/35 (77)	2
Atrial septal defect	5/16 (31)	11/35 (31)	0
Ventricular septal defect	3/17 (18)	3/35 (9)	0
Pulmonic stenosis	7/15 (47)	4/35 (11)	1
Patent ductus arteriosus	2/17 (12)	ND	ND
Mitral valve anomaly	5/17 (29)	8/32 (25)	2
Arrhythmia	6/16 (38)	8/9 (89)	ND
Others	TR 1, PH 1, atrioventricular valve dysplasia 1, valvular AS 1	polyvalvular dysplasia 2 pulmonary valve dysplasia 1, PFO 1, TOF 2, AS 1, right shaft deflection 1	
Skeletal/extremity deformity			
Cubitus valgus	2/9 (22)	7/22 (32)	2
Pectus deformity	5/13 (38)	20/31 (65)	2
Others		prominent finger pads 2	prominent finger pads 1
Skin/hair anomaly			
Curly hair	8/17 (47)	6/24 (25)	2
Hyperelastic skin	7/12 (58)	5/21 (24)	2
Café au lait spots	1/14 (7)	2/20 (10)	2
Lentigines	1/14 (7)	2/21 (10)	2
Naevus	3/15 (20)	9/22 (41)	0
Others	low posterior implantation 4, hyperpigmentation 3, redundant skin 3, sparse hair 2, sparse eyebrows 2, hemangioma 2	dry skin 3, sparse hair 3, sparse eyebrows 2, keratosis pilaris 2	
Genitalia	6/11 (55)	11/16 (69)	
Cryptorchidism	5/10 (50)	8/13 (62)	ND
Blood test abnormality			
Coagulation defects	2/11 (18)	1/4 (25)	ND

NS, Noonan syndrome; LS, LEOPARD syndrome; ND, not described; TR, tricuspid regurgitation; PH, pulmonary hypertension; AS, aortic stenosis; PFO, patent foramen ovale; TOF, tetralogy of Fallot.
[a]Includes affected family members. Clinical manifestations in 3, 21, and 11 NS patients with *RAF1* mutations were summarized from three reports [Ko et al., 2008; Pandit et al., 2007; Razzaque et al., 2007], respectively.

rather broad. However, Western blotting using antineomycin phosphoacetyltransferase antibody that recognizes the amount of plasmids introduced in cells showed that the transfection efficiency in cells expressing p.S427G was similar to that in cells expressing other mutants (Fig. 2A). These findings were consistently observed in three independent experiments. Recent studies have shown that autophosphorylation of S621 is required to prevent proteasome-mediated degradation [Noble et al., 2008]. To explore the possibility that p.S427G mutant is resistant to proteasome-mediated degradation, we examined the amount of WT RAF1 and p.S427G at 24, 48, and 72 hr after transfection in serum-starved or complete medium (Fig. 2C). The results showed that the expression of Myc-tagged RAF1 in cells expressing p.S427G was similar to that in WT RAF1, although multiple bands were observed, suggesting the hyperphosphorylation of the p.S427G mutant.

ELK Transactivation in Mutant RAF1 Proteins

To examine the effect on the downstream pathway of mutant RAF1, we introduced five RAF1 mutants into NIH3T3 cells and examined ELK transactivation (Fig. 2D). ELK is a transcription factor, which is phosphorylated by activated ERK and then binds the serum response element in the promoter of the immediate-early genes, including *C-FOS*. ELK transactivation was enhanced in cells expressing p.S257L, p.N262K, and p.S427G without any stimulation, suggesting that these mutants were gain-of-function

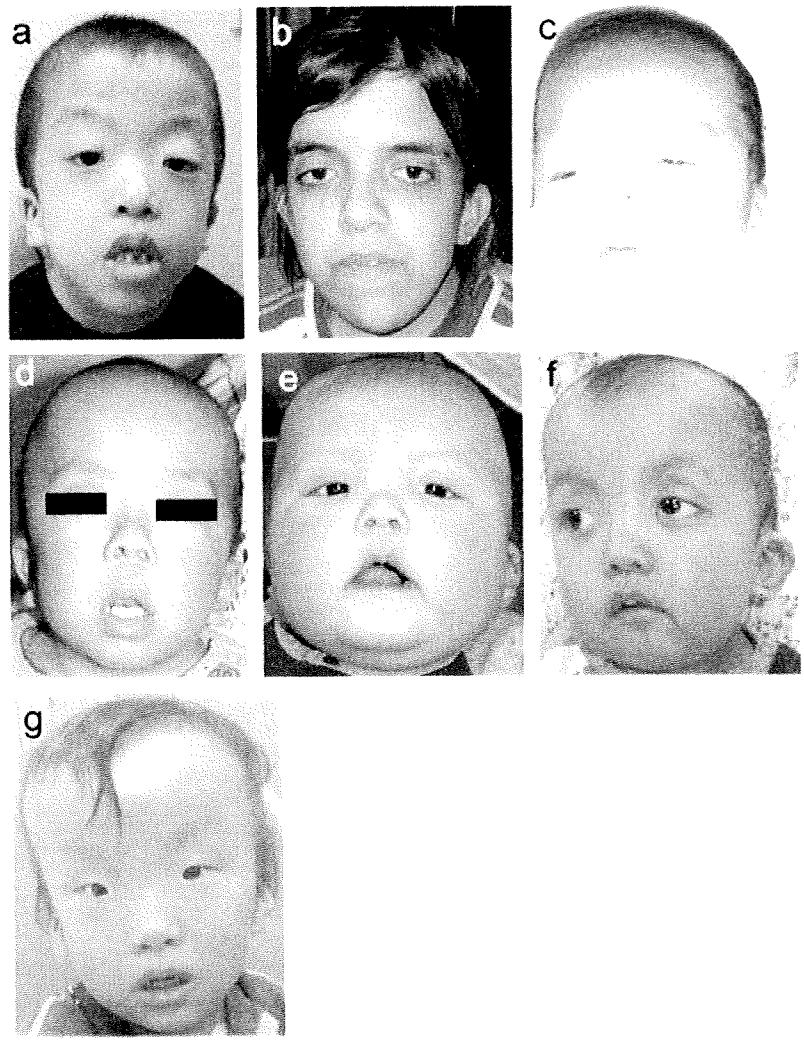

Figure 1. Facial appearance of patients with *RAF1* mutations. **a–f:** patients with p.S257L mutations. **a:** NS135; **b:** NS146; **c:** NS215; **d:** NS256; **e:** NS258 at 6 months; **f:** 2 years and 4 months; **g:** NS222 with p.S427G. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mutations. ELK transactivation in cells expressing p.H103G and p.R191I was not enhanced.

Phosphorylation State, ERK Activation, and Binding to the Scaffolding Protein 14-3-3 in Mutations in the CR2 Domain

Previous studies as well as the present study showed that mutations in NS-associated *RAF1* mutations were clustered in the CR2 domain. We hypothesized that amino acid changes in the CR2 domain impaired phosphorylation of serine at 259. We additionally generated expression construct harboring p.S259F and p.P261A substitutions, and their phosphorylation status was examined using anti-pRAF1 (S259) antibody together with RAF1 WT, p.S257L, p.N262K, and p.S427G (Fig. 3A). The results showed that phosphorylated proteins were scarcely observed in p.S257L, p.S259F, p.P261A, and p.N262K. Phosphorylation of ERK p44/42 was determined using anti-p-ERK (p44/42) antibody. All mutations activated the downstream ERK without any stimulation. The level of ERK phosphorylation in cells expressing mutants was lower than that in those treated with epidermal growth factor (EGF), suggesting that the expression of p.S257L, p.S259F, p.P261A, and p.N262K resulted in a partial activation of ERK.

Anti-pRAF1 (S259) antibody was produced by immunizing rabbits with a synthetic phospho-peptide corresponding to residues surrounding Ser259 of human RAF1. To examine if this antibody was able to recognize phosphorylation at S259 when mutations such as S257L and N262K were introduced, we performed a solid-phase immunoassay using biotinylated peptides as per the manufacturer's recommendation (Mimotopes, Victoria, Australia; Supp. Methods). The result showed that at least in peptides, this antibody could recognize serine phosphorylation in amino acid 259 when mutations S257L and N262K were introduced (Fig. 3B). These results support the data in Figure 3A, suggesting that S259 was not phosphorylated in mutants in the CR2 domain.

To examine if the RAF1 mutants without S259 phosphorylation were able to bind to 14-3-3, we cotransfected three double mutants (WT/S621A, S257L/S621A, and N262K/S621A) with FLAG-tagged 14-3-3, and coimmunoprecipitation was performed using anti-Myc antibody (Fig. 3C). The result showed that the WT/S621A mutant bound 14-3-3. In contrast, p.S257L/S621A and

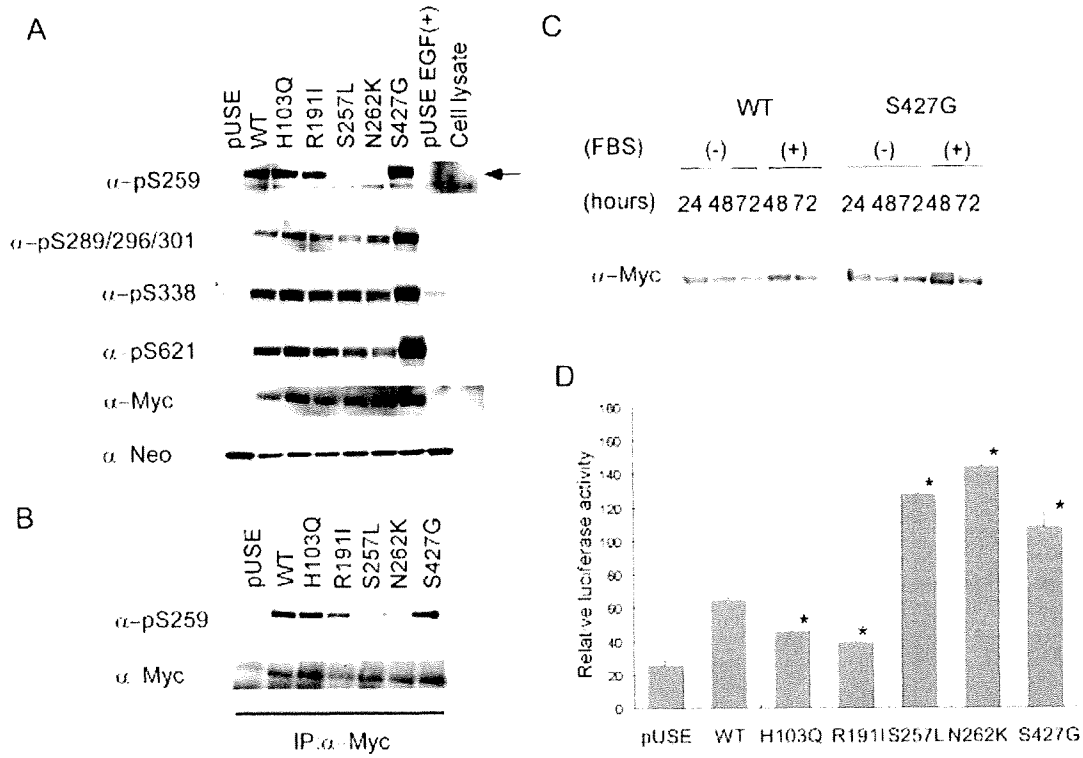

Figure 2. Analysis of phosphorylation status, degradation, and effect on downstream signaling in RAF1 mutants identified in this study. **A:** Phosphorylation status of wild-type (WT) RAF1 and mutants. Expression levels of RAF1 proteins and their phosphorylation levels were detected with different antibodies indicated in the figure. Transfection efficiency was measured using antineomycin phosphotransferase II (α-Neo) antibody. The arrow indicates the serine-phosphorylated expressed RAF1. **B:** Phosphorylation of S259 was confirmed by immunoprecipitation. Myc-tagged RAF1 was immunoprecipitated using anti-Myc antibody and the phosphorylation of S259 was determined. **C:** Time course experiments of WT RAF1 and p.S427G. The RAF1 protein was detected using anti-Myc antibody (clone 4A6; Millipore). FBS, fetal bovine serum. **D:** ELK transactivation in WT and mutants. Results are expressed as the means and standard deviations of mean values from triplicate samples. A significant increase in relative luciferase activity (RLA) was observed in cells transfected with p.S257L, p. N262K, and p.S427G, but not in cells transfected with p.H103Q or p.R191I. WT, wild-type; $^{*}P<0.01$ by Student's *t*-test.

p.N262K/S621A mutants did not bind 14-3-3, suggesting that the decreased phosphorylation of S259 prevented 14-3-3 binding. A similar result was obtained in the coimmunoprecipitation study using anti-FLAG antibody (Fig. 3D). These results showed that mutants in the CR2 domain impaired phosphorylation of S259, abrogated the binding to 14-3-3 and resulted in a partial activation of ERK.

Discussion

In this study, we identified eight different *RAF1* mutations in 18 patients: p.S257L in 11 patients and p.R191I, p.S259F, p.P261A, p.P261L, p.N262K, p.S427G, and p.L613V in one patient each. Sixteen patients were diagnosed as having NS, although we were not able to reevaluate 2 patients with Costello syndrome. Examination of detailed clinical manifestations in the present study and past studies showed that patients with *RAF1* mutations were associated with hypertrophic cardiomyopathy, arrhythmia, and mental retardation. Results from previous studies and the present study showed 41/52 (79%) mutations to be located in the CR2 domain (Fig. 3E). We first demonstrated that mutations in the CR2 domain had impaired phosphorylation of S259. This caused the impaired binding of RAF1 to 14-3-3, resulting in a partial activation of downstream ERK. These results suggest that dephosphorylation of S259 is the primary mechanism of activation of mutant RAF1 located in the CR2 domain.

Phosphorylation of S259 and subsequent binding to 14-3-3 have been shown to be important for suppression of RAF1 activity [Dhillon et al., 2007]. Light et al. [2002] examined the phosphorylation status at S259 in the p.S257L mutant. Their experiment showed that phosphorylation of S259 still existed in the p.S257L mutant. The mutant was not able to bind 14-3-3 [Light et al., 2002]. In contrast, our functional studies demonstrated that all four mutants located in the CR2 domain (p.S257L, p.S259F, p.P261A, and p.N262K) impaired phosphorylation of S259 and that two of them impaired binding of 14-3-3. Impaired binding to 14-3-3 was also shown in p.P261S mutant [Pandit et al., 2007]. The reason for the difference on S259 phosphorylation between the result by Light et al. [2002] and ours is unclear. Enhanced kinase activities of mutants, including p.S257L, p.P261S, p.P261A, and p.V263A, were demonstrated in a previous study [Razzaque et al., 2007]. Phosphorylation levels at S338 in p.S257L and p.N262K were not enhanced compared to that in WT RAF1 (Fig. 2A), suggesting that the activation mechanism in these mutants is different from that of the normal state upon RAS-GTP binding. Indeed, ERK activation was partial compared with that in cells after EGF treatment (Fig. 3A). These results suggest that the conformational change around S259 due to amino acid changes results in the decreased phosphorylation of S259 and that mutant

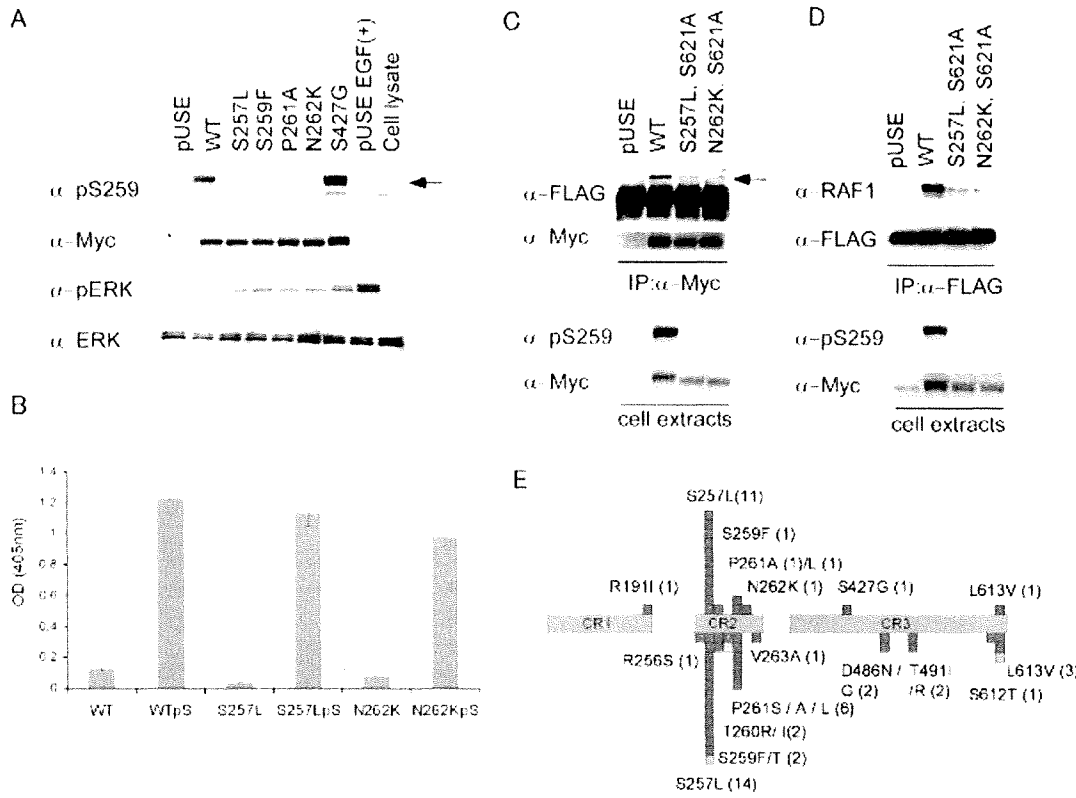

Figure 3. Phosphorylation of S259, binding to 14-3-3 and ERK activation of mutants located in the CR2 domain. **A:** Phosphorylation status of WT and mutants located in the CR2 domain. Phosphorylation of S259 was not observed in cells expressing p.S257L, p.S259F, p.P261A, and p.N262K. In order to examine the level of full activation of ERK, mock-transfected cells were treated with 10 ng/ml EGF. ERK activation was observed in cells expressing p.S257L, p.S259F, p.P261A, and p.N262K, but was weaker than those in cells expressing p.S427G and EGF-treated cells. The arrow indicates the serine-phosphorylated expressed RAF1. **B:** Epitope mapping of the anti-pRAF1 (S259) antibody using a solid-phase immunoassay. The antibody was able to recognize peptides with S257L or N262K mutations when S259 was phosphorylated, but was not able to recognize peptides without Ser259 phosphorylation. Results are expressed as the means and standard deviations of mean values from triplicate samples. **C:** Binding of RAF-1 to 14-3-3ζ. HEK293 cells were transfected with constructs harboring FLAG-tagged 14-3-3 and one construct of pUSE WT, p.S257L/p.S621A, or p.N262K/ p.S621A. Immunoprecipitation was performed using anti-Myc antibody, and 14-3-3 binding was determined by anti-FLAG antibody (upper panel). Phosphorylation of S259 and RAF1 expression were determined in cell lysates used for the immunoprecipitation (lower panel). The arrow indicates the band for 14-3-3. **D:** Binding of 14-3-3ζ to RAF-1. Immunoprecipitation was performed using anti-FLAG antibody and RAF1 binding was examined using anti-RAF1 antibody (upper panel). The binding of 14-3-3 to endogenous RAF1 was scarcely observed (lane 1, pUSE). Phosphorylation of S259 and RAF1 expression were determined using cell lysates used for the immunoprecipitation (lower panel). **E:** Domain organization and the distribution of mutations in RAF1 protein. The three regions conserved in all RAF proteins (conserved region [CR] 1, CR2, and CR3) are shown in pink. Mutations identified in this study are shown above the bar and those reported before [Ko et al. 2008; Pandit et al. 2007; Razzaque et al. 2007] are shown below the bar. Green squares indicate families with NS. Orange squares indicate patients with LEOPARD syndrome and the yellow square indicates a patient with hypertrophic cardiomyopathy.

RAF-1 then dissociates from 14-3-3; the substrate would thus be targeted to the catalytic domain in the CR3 domain (Fig. 4).

To highlight the clinical pictures of patients with *RAF1* mutations, clinical manifestations in 52 patients with *RAF1* mutations [Ko et al., 2008; Pandit et al., 2007; Razzaque et al., 2007], 172 patients with *PTPN11* mutations [Jongmans et al., 2005; Musante et al., 2003; Tartaglia et al., 2002; Zenker et al., 2004], 73 patients with *SOS1* mutations [Ferrero et al., 2008; Narumi et al., 2008; Roberts et al., 2007; Tartaglia et al., 2007; Zenker et al., 2007a] and 18 patients with *KRAS* mutations [Carta et al., 2006; Ko et al., 2008; Lo et al., 2008; Schubbert et al., 2006; Zenker et al., 2007b] are summarized in Table 3. The frequency of perinatal abnormalities was similar between patients with *RAF1* and *SOS1*. In contrast, the description of perinatal abnormalities was rare in patients with *PTPN11* and *KRAS* mutations. Growth failure and mental retardation were observed in 100 and 94% of NS with *KRAS* mutations, respectively. Growth failure and mental retardation were observed in 87 and 56% of patients with *RAF1* mutations, respectively. In contrast, those manifestations were less frequent (63 and 43%) in patients with *PTPN11* mutations. The frequency of mental retardation was lowest in patients with *SOS1* mutations (18%). We were unable to compare gene-specific features in craniofacial characteristics because such details were not described in the previous reports. As for skeletal characteristics, short stature was frequently manifested in patients with *RAF1* mutations (82%) followed by *KRAS* mutation-positive patients (71%). The association of short stature was lower in *PTPN11* mutation-positive and *SOS1* mutation-positive patients (56 and 38%, respectively). It is noteworthy that the association of hypertrophic cardiomyopathy was specifically high (73%) in *RAF1* mutation-positive patients. In contrast, hypertrophic cardiomyopathy was observed in 20% of clinically diagnosed Noonan

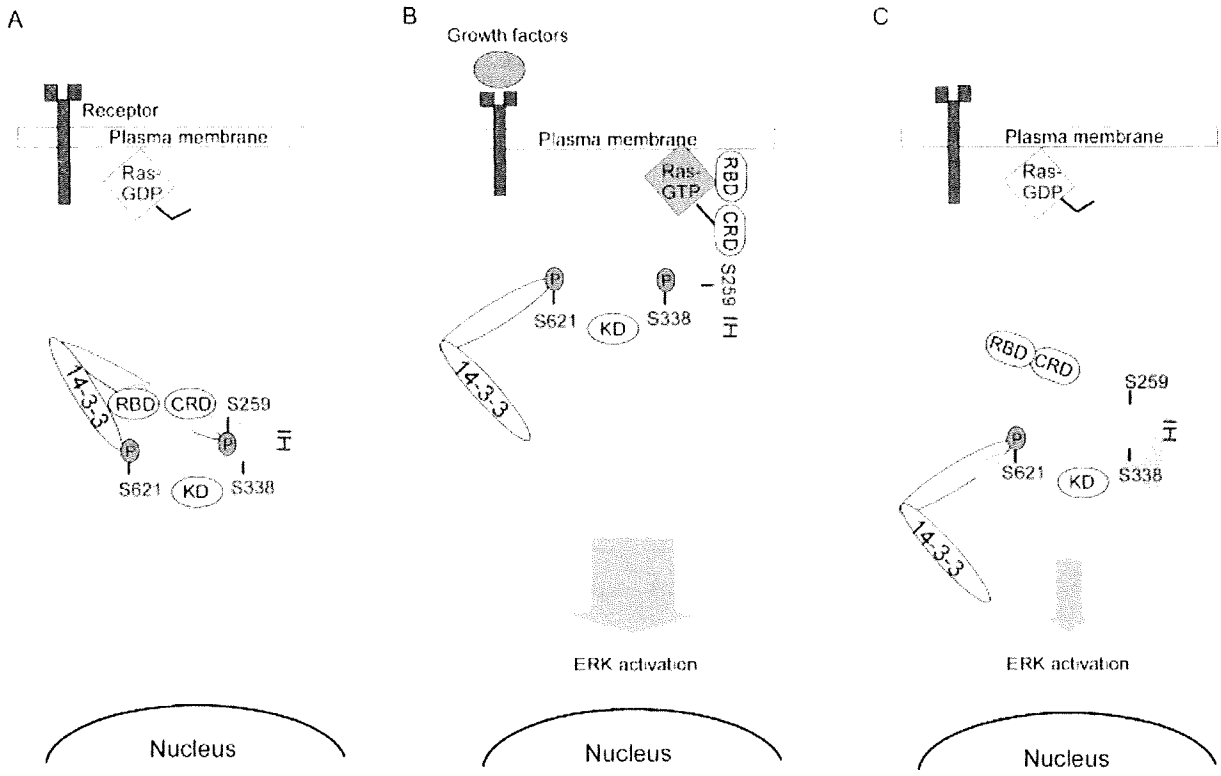

Figure 4. Schematic model of WT and mutant activation. **A:** In an inactive state, RAF1 is phosphorylated on S259 and S621 and is bound to 14-3-3. **B:** In growth-factor stimulation, the GTP-bound RAS binds to the CR1 domain of RAF1, which displaces 14-3-3. S259 is dephosphorylated by protein phosphatase 1 (PP1) and/or protein phosphatase 2A (PP2A). After RAF1 is recruited to the plasma membrane, phosphorylation of S338, Y341, T491, and S494 occurs. The phosphorylation of these residues is thought to be important for the full activation of RAF1. **C:** Mutants whose amino acid changes are located in the CR2 domain. It has been reported that S259 was phosphorylated by Akt and dephosphorylated by PP1 and/or PP2A. Amino acid changes in the CR2 domain would cause structural changes in the CR2 domain, leading to the access of PP2A to S259. Alternatively, Akt kinase would not be able to phosphorylate S259. S259 is dephosphorylated without stimulation and substrate(s) would be able to enter the kinase domain, leading to a partial activation. RBD, RAS-binding domain; CRD, cysteine-rich domain; KD, kinase domain; IH, isoform-specific hinge segment region. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

patients [van der Burgt 2007] and in 7, 10, and 17% of patients with *PTPN11*, *SOS1*, and *KRAS* mutations, respectively. These results strongly suggest that patients with *RAF1* mutations have a significantly higher risk of hypertrophic cardiomyopathy. Mitral valve abnormality and arrhythmia were also frequently observed in patients with *RAF1* mutations (27 and 56%, respectively). In summary, these results highlight specific manifestations of patients with *RAF1* mutations: high frequency of hypertrophic cardiomyopathy, septal defects of the heart, short stature, and less frequent PS (Supp. Fig. S1). The high frequency of heart defects would be associated with a high risk of sudden death in *RAF1* mutation-positive patients.

The present study is the first to identify p.S427G in a patient with NS. The same mutation has been reported in a patient with therapy-related acute myeloid leukemia [Zebisch et al., 2006]. The patient reported by Zebisch et al. [2006] first developed immature teratoma, yolk sack tumor, and embryonal testicular carcinoma. Thirty-five months after tumor resection and chemotherapy, the patient developed acute myeloid leukemia. Molecular analysis of *RAF1* revealed the de novo p.S427G mutation in leukemia cells and DNA from buccal epithelial cells [Zebisch et al., 2006]. Whether or not the patient had an NS phenotype was not mentioned. *RAF1* mutations have been rarely reported in malignant tumors. As far as we could determine, only six mutations, including p.P207S, p.V226I, p.Q335H, p.S427G, p.I448V, and p.E478K, have been identified in tumors and therapy-related leukemias [Pandit et al., 2007; Razzaque et al., 2007]. A previous study as well as our results showed that p.S427G mutant has transformation capacity [Zebisch et al., 2009], is resistant to apoptosis when introduced into NIH3T3 cells [Zebisch et al., 2009] and activates ERK and ELK transcription, suggesting that p.S427G is a gain-of-function mutation. We identified p.S427G in a familial case of NS. The mother and boy have not yet developed malignant tumors. Although no NS patients with *RAF1* mutations have developed malignant tumors, careful observation might be prudent in *RAF1* mutation-positive children.

We identified two novel mutations, p.R191I and p.N262K. p.R191I is located in the CR1, and arginine at amino acid position 191 is evolutionally conserved [Mercer and Pritchard, 2003]. Activation of ERK was not observed in cells expressing p.R191I. ELK transactivation was rather decreased; parental samples were not available. There is a possibility that this change is a polymorphism.

In conclusion, we identified *RAF1* mutations in 18 patients and detailed clinical manifestations in mutation-positive patients were examined. Our analysis of patients with mutations in *RAF1*, *PTPN11*, *SOS1*, and *KRAS* showed hypertrophic cardiomyopathy and short stature to be frequently observed in patients with *RAF1* mutations. Functional analysis revealed that dephosphorylation of S259 would be the essential mechanism for ERK activation in RAF1 mutations. Despite recent progress in molecular characterization of NS and related disorders, genetic causes in

Table 3. Clinical Manifestations in NS Patients with *RAF1*, *PTPN11*, *SOS1*, and *KRAS* Mutations

	RAF1[a] (%)	PTPN11[b] (%)	SOS1[c] (%)	KRAS[d] (%)
Total patients	52	172	73	18
Perinatal abnormality				
Polyhydramnios	12/34 (35)	ND	9/16 (56)	2
Fetal macrosomia	11/31 (35)	ND	9/15 (60)	ND
Growth and development				
Failure to thrive in infancy	13/15 (87)	35/56 (63)	ND	3/3 (100)
Mental retardation	25/45 (56)	71/164 (43)	12/67 (18)[e]	16/17 (94)[f]
Outcome				
Died	6/28 (21)	ND	ND	ND
Craniofacial characteristics				
Relative macrocephaly	32/38 (84)	ND	9/21 (43)[e]	9/11 (82)
Hypertelorism	34/36 (94)	15/28 (54)[e]	5/6 (83)	12/12 (100)
Downslanting palpebral fissures	29/37 (78)	19/28 (68)	20/22 (91)	9/12 (75)
Ptosis	28/37 (76)	18/29 (62)	19/24 (79)	10/15 (67)
Epicanthal folds	24/35 (69)	15/28 (54)	ND	2/9 (22)[e]
Low set ears	32/36 (89)	56/64 (88)	20/22 (91)	7/10 (70)
Skeletal characteristics				
Short stature	41/50 (82)	97/172 (56)[e]	22/58 (38)[e]	12/17 (71)
Short neck	35/46 (76)	15/29 (52)[e]	17/22 (77)	9/10 (90)
Webbing of neck	38/46 (83)	36/122 (30)[e]	3/6 (50)	7/14 (50)[e]
Cardiac defects				
Hypertrophic cardiomyopathy	37/51 (73)	10/135 (7)[e]	7/73 (10)[e]	3/18 (17)[e]
Septal defect	22/52 (42)	41/170 (24)[e]	17/73 (23)[e]	5/18 (28)
Atrial septal defect	16/51 (31)			4/18 (22)
Ventricular septal defect	6/52 (12)			1/18 (6)
Pulmonic stenosis	11/50 (22)	125/171 (73)[f]	53/73 (73)[f]	7/18 (39)
Patent ductus arteriosus	2/20 (10)	ND	ND	1/18 (6)
Mitral valve anomaly	13/49 (27)	ND	ND	3/18 (17)
Arrhythmia	14/25 (56)	ND	ND	ND
Skeletal/extremity deformity				
Cubitus valgus	9/31(29)	14/61 (23)	1/6 (17)	2/2 (100)
Pectus deformity	25/44 (57)	108/171 (63)	38/56 (68)	13/16 (81)
Skin/hair anomaly				
Curly hair	14/41 (34)	ND	15/22 (68)[f]	1/12 (8)
Hyperelastic skin	12/33 (36)	ND	1/6 (17)	3/12 (25)
Café au lait spots	3/34 (9)	ND	1/6 (17)	1/9 (11)
Lentigines	3/35 (9)	ND	ND	ND
Naevus	12/37 (32)	ND	ND	ND
Genitalia				
Cryptorchidism	13/23 (57)	75/138 (54)	22/39 (56)	4/11 (36)
Blood test abnormality				
Coagulation defects	3/15 (20)	46/90 (51)	14/66 (21)	2/9 (22)

ND, not described.
[a][Ko et al., 2008; Pandit et al., 2007; Razzaque et al., 2007]; and this study.
[b][Jongmans et al., 2005; Musante et al., 2003; Tartaglia et al., 2002; Zenker et al., 2004].
[c][Ferrero et al., 2008; Ko et al., 2008; Narumi et al., 2008; Roberts et al., 2007; Tartaglia et al., 2007; Zenker et al., 2007a].
[d][Carta et al., 2006; Ko et al., 2008; Lo et al., 2008; Schubbert et al., 2006; Zenker et al., 2007b].
[e]The frequency of the manifestation in patients with the gene was significantly lower compared with that observed in *RAF1*-positive patients ($P<0.05$ by Fisher's exact test).
[f]The frequency of the manifestation in patients with the gene was significantly higher compared with that observed in *RAF1*-positive patients ($P<0.05$ by Fisher's exact test).

approximately 30% of NS and related disorders remain unknown. Presently unknown genetic causes for mutation-negative NS and related disorders remain to be identified in molecules in future studies.

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CONFERENCE REPORT

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Proceedings From the 2009 Genetic Syndromes of the Ras/MAPK Pathway: From Bedside to Bench and Back

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The RASopathies are a group of genetic syndromes caused by germline mutations in genes that encode components of the Ras/mitogen-activated protein kinase (MAPK) pathway. Some of these syndromes are neurofibromatosis type 1, Noonan syndrome, Costello syndrome, cardio-facio-cutaneous syndrome, LEOPARD syndrome and Legius syndrome. Their common underlying pathogenetic mechanism brings about significant overlap in phenotypic features and includes craniofacial dysmorphology, cardiac, cutaneous, musculoskeletal, GI and ocular abnormalities, and a predisposition to cancer. The proceedings from the symposium "Genetic Syndromes of the Ras/MAPK Pathway: From Bedside to Bench and Back" chronicle the timely and typical research symposium which brought together clinicians, basic scientists, physician-scientists, advocate leaders, trainees, students and individuals with Ras syndromes and their families. The goals, to discuss basic science and clinical issues, to set forth a solid framework for future research, to direct translational applications towards therapy and to set forth best practices for individuals with RASopathies were successfully meet with a commitment to begin to move towards clinical trials.

Key words: cardio facio-cutaneous syndrome; clinical trial; Costello syndrome; neurofibromatosis type 1; Noonan syndrome; Legius syndrome; Ras/MAPK; signal transduction pathway; RASopathy; therapy

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INTRODUCTION

The symposium "Genetic Syndromes of the Ras/MAPK Pathway: From Bedside to Bench and Back" was held on August 1–2, 2009 in Berkeley, CA at the Doubletree Hotel & Executive Meeting Center at the Berkeley Marina. The topic of this timely symposium was the "RASopathies," a class of developmental disorders caused by dysregulation of the Ras/mitogen-activated protein kinase (MAPK) pathway [Tidyman and Rauen, 2009]. The main focus of this conference was on five syndromes associated with the pathway: Noonan syndrome (NS), Costello syndrome (CS), cardio-facio-cutaneous syndrome (CFC), neurofibromatosis type 1 (NF1), and Legius syndrome which share phenotypic features including distinctive craniofacial features, cardiovascular anomalies, musculoskeletal and cutaneous abnormalities, neurocognitive delay and, in some syndromes, cancer. This unprecedented NIH-sponsored symposium (http://cancer.ucsf.edu/raspathway2009/index.php) was held in conjunction with family conferences sponsored by the Noonan Syndrome Support Group (NSSG), the Costello Syndrome Family Network (CSFN) and the International Costello Syndrome Support Group (ICSSG), CFC International and two neurofibromatosis support groups, NF Inc. California and the Children's Tumor Foundation (CTF). This symposium brought together clinicians, basic scientists, physician-scientists, advocate leaders, trainees, students and individuals with Ras/MAPK syndromes and their families. Nearly 200 registrants participated. The symposium was supported by an R13 grant (HD061140) obtained through the National Institutes of Heath with contributing institutes including the National Institute of Child Health and Human Development, the National Cancer Institute and the Office of Rare Diseases Research. Additional educational funds were provided by Novo Nordisk, UC Discovery, UCSF Clinical Translational Science Institute, the UCSF Helen Diller Family Comprehensive Cancer Center, the UCSF Children's Hospital, Schering-Plough and GeneDx. The symposium achieved its goal to provide an open forum for researchers, clinicians and physician-scientists to share and discuss basic science and clinical issues setting forth a solid framework for future research, translational applications directed towards therapy and best practices for individuals with RASopathies. The purposes of these proceedings are to provide the clinical and scientific communities with an executive summary of the research symposium and to publish the abstracts from the platform presentations and poster session.

SUMMARY OF PRESENTATIONS

This inaugural research symposium began with two special keynote addresses. "The Road Less Traveled: Our Journey on the Ras/MAPK Pathway" was a composite video presentation created by parent advocates from the NSSG, the ICSSG/CSFN, NF Inc. California and CFC International. The presentation provided perspectives of families with Ras syndromes, sharing the concerns and issues associated with activities of daily living. The video presentation also expressed the cautious optimism of the potential within each individual affected by a Ras/MAPK pathway mutation. The second keynote address "New Perspectives on an Ancient Pathway," by Frank McCormick, provided historical and scientific perspectives of Ras and the Ras pathway focusing on the trials and tribulations of cancer therapeutic development.

Session I, "The Clinical Consequences of Molecular Alterations," led by Angela Lin, reviewed the phenotypic features of NS, NF1, CFC, CS, and Legius syndrome. Each speaker reviewed the molecular alterations, and the cardinal features which are frequently overlapping. These features included variable neurocognitive impairment, skin and cardiac anomalies, and often, a characteristic facial appearance. The final presentation highlighted the extensive

phenotypic overlap of the RASopathies and the challenges in naming syndromes, in general. An axis-based innovative approach to future classification was recommended.

Session II "Biochemical Properties and Function of Altered Proteins: Perturbation of the Pathway," led by Eric Legius, discussed the functional consequences of the mutations in the different genes of the pathway observed in patients. Hereditary mutations of genes coding for proteins that are components of the Ras/MAPK pathway can activate the pathway by different mechanisms of pathway activation: (1) inactivating mutations in negative regulators of the pathway such as neurofibromin (NF1) or SPRED1 (Legius syndrome) and (2) activating mutations as seen in CS, NS, LEOPARD, and CFC. The mutations observed in these genes are usually missense resulting in protein activation with subsequent hyperactivation of the pathway. Some mutations do not result in a functional activation of the resulting protein "in vitro," but nevertheless are found in patients with the same, or similar clinical features as patients with typical kinase activating mutations.

Session III entitled "Pre-Clinical Modeling of Ras/MAPK Pathway Syndromes," which was moderated by Bruce Gelb, focused on translational research with genetically engineered animal models. The session began with a presentation on the current preclinical trials consortium using mouse models of *Nf1* to assess novel therapeutic agents as a potential model for developing therapies for other inherited Ras pathway disorders. Several of the presentations described advances in understanding the pathogenesis of disease for Ras/MAPK pathway defects, primarily by expressing germline or somatic gain-of-function mutant proteins in a tissue-specific manner.

Session IV, "Application of New Technologies" moderated by Yoko Aoki discussed new technologies which could be considered to advance the knowledge of the RASopathies. The discussion included (1) the application of novel network approaches including expression Quantitative Trait Locus (eQTL) to identify signaling hubs that may be important targets for drug development or gene interaction, (2) the application of clinical proteomics for analysis and characterization of proteins and peptides present in body fluids, cells or tissues, and (3) novel phospho-signaling networks and stochastic modeling of phenotypic markers.

Session V entitled "Potential Therapeutic Options: Modulating the Pathway" was lead by Martin Zenker. The Ras pathway has been highly studied in the context of cancer with many inhibitors in development, or in clinical trials; some are FDA approved. The basic mechanism of action of FTIs, as well as Raf and MEK inhibitors were discussed along with the possibility of "re-purposing" such inhibitors to treat individuals with RASopathies long term. Current efforts in the development of Ras pathway inhibitors as anticancer drugs are focusing on the improvement of selectivity as well as on strategies to avoid possible paradoxical effects that may be related to feedback loops within the Ras pathway. In vivo application of inhibitors was presented for NF1, NS, and CFC models.

Session VI, "Moving Forward: Treating Genetic Syndromes" was moderated by David Viskochil and explored treatment for genetic syndromes. At present, a clinical trial for Hutchinson-Gilford progeria using a FTI is underway. Discussion of this clinical trial provided a framework for the development and implementation of clinical trials for children with extremely rare genetic conditions. Key issues included the development of a unified clinical database for all affected individuals before the actual trial, and from that information the mindful selection of outcome measures. NF1 was discussed as a model for the development and organization of a multi-center clinical trials consortium. The treatment of plexiform neurofibroma progression with an mTOR inhibitor was presented, and included discussion on the selection of endpoints and recognition of windows of opportunity for effective treatment. Fragile X syndrome is another rare genetic disorder undergoing clinical trials. An mGluR5 antagonist, fenobam, is used for behavior as measured by prepulse inhibition and minocycline is used for language and attention therapy in fragile X individuals. The importance of selecting measurable endpoints of treatment was emphasized.

The Wrap-Up Session closing the symposium was moderated by Roger Packer and Teri Melese. The conduction of clinical trials within consortia, and the critical issue of collaborations between academic centers, biotechnology companies and pharmaceutical companies were discussed. The federal initiative through the Office of Rare Diseases Research (ORDR) for Therapeutics for Rare and Neglected Diseases (TRND) was reviewed in the context of developing a Clinical Trials Consortium to develop rational protocols and implement trials for the Ras Pathway disorders. The symposium ended with a commitment from participants to organize further formal discussions on treatment.

SPEAKERS' ABSTRACTS

New Perspectives on an Ancient Pathway

Frank McCormick. The Ras/MAPK pathway is one of the best-trodden in cancer biology. The discovery that Ras genes are mutated in human cancer dates back almost 30 years, while the relationship between Ras, Raf, Mek, and Erk was established some 10 years later, at the same time that the *NF1* gene was identified and characterized. These early discoveries prompted attempts to identify drugs that would block the pathway and be effective in treating cancers and NF1, and led to the development of small molecules that inhibit Raf kinase or Mek kinase, amongst other targets in the pathway. More recently, the role of the PI 3′ kinase pathway in Ras signaling has been better appreciated, prompting drug discovery in this pathway, and the role of this pathway in other diseases, such as CS, NS, CFC, and others (The RASopathies) has been appreciated. The Ras pathway, generally defined, is highly regulated. Indeed the consequences of mis-regulation are strikingly clear from the phenotypes associated with cancer and RASopathies. Attempts to block these pathways with drugs have often led to failure, due to back-up systems and feedback loops. Blockade of Ras by FTIs was thwarted by geranylgeranyl transferase activity that restored Ras activity following de-farnesylation. Feedback loops regulate signal transmission from Ras at multiple levels. ERK phosphorylates receptor tyrosine kinases, Sos, and Raf kinases, for example. In tumor cells harboring activated alleles of Ras or B-Raf, these feedback loops are expected to hyperactivated also. Conversely, when kinases in this pathway are blocked by pharmacologic agents, loss of feedback is likely to hyper-activate upstream elements, with important consequences for therapy. Sorafenib, a drug approved for treatment of

advanced hepatocellular carcinoma is more effective on tumors with high levels of activated phospho-ERK, consistent with its ability to inhibit Raf kinase activity. While these tumors do not contain mutations in RAS or RAF genes, high levels of Raf kinase signaling have been reported, through loss of function of Sprouty/SPRED proteins. On the other hand, Sorafenib failed to show clinical activity in melanomas expressing V600E BRAF, possibly because of altered feedback loops in these cells. Likewise, MEK inhibitors that appeared potent and effective in preclinical models have failed, so far, in the clinic, possibly because of unrecognized feedback loops and back-up systems that diminish their effects in vivo. We recently described a novel feedback loop involving ephrin signaling. Engagement of the EphA2 receptor by ephrins presented on neighboring cells results in negative regulation of the Ras/MAPK pathway by a novel mechanism. New data relating to the role of GAPs in this process will be discussed, including biochemical analysis of the neurofibromin and Sprouty/SPRED protein. Analysis of these complex and dynamic properties of the ancient, well-trodden pathway has led to new insights into how signals are transmitted, regulated and terminated, and new ways of predicting effects of blocking individual elements of the pathway. Analysis of cancer cells and RASopathies has played an important role in understanding these processes at the molecular level, and, hopefully, will lead to better ways of developing effective and safe therapies.

Clinical Features of Noonan Syndrome

Judith Allanson. NS is a common autosomal dominant multiple congenital anomaly syndrome first described over 40 years ago. The incidence is reported to be between 1 in 1,000 and 1 in 2,500, although mild expression may be more common. The cardinal features of NS are well delineated. They include short stature, congenital heart defects (particularly pulmonary valve dysplasia) and/or hypertrophic cardiomyopathy, broad or webbed neck, chest deformity with pectus carinatum superiorly and pectus excavatum inferiorly, developmental delay of variable degree, cryptorchidism, coagulation defects and lymphatic dysplasias. The facial appearance of NS is well established and shows considerable change with age, being most striking in the newborn period and middle childhood and most subtle in the adult. Many adults have features that differ little from those in the general population. Other adults are recognizable because of classical facial features, which include ptosis and wide-spaced eyes, low set, posteriorly rotated ears with a fleshy helix, inverted triangular face which is broad at the temples and tapers to a small chin, and long and broad or webbed neck.

Neurofibromatosis Type 1 and Legius Syndrome

David A. Stevenson. *NF1*: NF1 is an autosomal dominant condition due to mutations in the *NF1* gene, which encodes neurofibromin, a negative regulator of Ras signaling. Variable expressivity is common even within families and the manifestations are age-dependent. In the 1980s a group of experts decided on clinical diagnostic criteria for NF1 and includes the presence of two of the following: (1) six or more café au lait macules over 5 mm in it greatest diameter in prepubertal individuals and over 15 mm in its greatest diameter in postpubertal individuals; (2) two or more neurofibromas of any type or one plexiform neurofibroma; (3) freckling in the axillary or inguinal regions; 4) two or more Lisch nodules (iris hamartomas); (5) optic glioma; (6) a distinctive osseous lesion such as sphenoid dysplasia or thinning of long bone cortex, with or without pseudarthrosis; (7) first-degree relative (parent, sibling, or offspring) with NF-1 by the above criteria. The clinical diagnostic criteria have historically been useful for the clinician for diagnostic purposes although other manifestations are not included in the diagnostic criteria (e.g., macrocephaly, malignant peripheral nerve sheath tumors, learning disorders). A small subset of individuals fulfills the NF1 clinical diagnostic criteria with café au lait macules and freckling in the axillary or inguinal regions without the development of neurofibromas or Lisch nodules. A cohort of individuals with a documented 3-bp in-frame deletion in exon 17 (c.2970_2972 delAAT) provides what appears to be a unique *NF1* genotype–phenotype relation. *Legius syndrome:* Recently, individuals with pigmentary findings fulfilling NF1 clinical diagnostic criteria (but without tumor development) did not have an identifiable *NF1* mutation, but instead harbored mutations in the *SPRED1* gene. *SPRED1* negatively regulates the Ras/MAPK signaling pathway at the Ras–Raf interface. Other clinical features reported in this cohort included macrocephaly, "Noonan-like face," lipomas, and learning problems. *Conclusion:* The currently utilized diagnostic criteria for NF1 may be insufficient to differentiate NF1 from Legius syndrome. Mutation analysis of *NF1* and *SPRED1* can help delineate these overlapping syndromes and potentially change clinical management schemes, although more information on the clinical phenotype of Legius syndrome will be required to sort out differences in anticipatory guidance and screening regimens from NF1. Future studies to assess cost-effectiveness of mutation analysis will be needed. The clinical overlap between Legius syndrome and NF1 makes inclusion of individuals in NF1 clinical research protocols based solely on NIH diagnostic criteria problematic.

Costello Syndrome

Karen W. Gripp. CS is one of several Ras/MAPK pathway disorders, collectively referred to as "RASopathies." While RASopathies share many phenotypic features, and the clinical differential diagnosis can be particularly challenging in young patients, CS has a distinctive phenotype. The pregnancy of a fetus with CS is typically complicated by polyhydramnios (92%) and macrocephaly (50%), and delivery is preterm (50%) to parents with advanced paternal age (62%), while fetal tachyarrhythmia is less common. Postnatally, severe feeding difficulties and failure to thrive typically necessitate feeding tube placement. Weight and height remain below normal, whereas the head circumference is within the normal range, resulting in relative macrocephaly. Hypotonia and developmental delay are noted from infancy, and standardized cognitive testing in older individuals shows the mean full scale IQ to be in the range of mild mental retardation (56–69), with particular weakness in expressive language. Facial features coarsen over time and include a wide mouth with prominent lips, and wart-like lesions termed papillomata may occur. Deep palmar and plantar creases, with redundant soft tissue and loose small joints are typical. Ulnar deviation of the wrists and fingers is characteristic for CS. Almost all individuals

develop tight Achilles tendons necessitating surgical release. Progressive kyphoscoliosis and osteopenia may affect teenagers and young adults. Growth hormone deficiency is common, and pubertal development is often dysregulated. Cardiac involvement is noted in about 3/4, including cardiac hypertrophy (41%), congenital heart defect (21%), and supraventricular tachycardia (33%). CS is a tumor predisposition syndrome with a 15% lifetime risk for a malignancy, with rhabdomyosarcoma being the most common (60%), followed by neuroblastoma, and bladder cancer. Rhabdomyosarcoma and neuroblastoma are childhood cancers and occur in the typical age range in CS. In contrast, bladder cancer is typically a malignancy of older adults, but may occur as early as the second decade of life in individuals affected by CS. Germline mutations in the *HRAS* gene are the only genetic cause of CS. *HRAS* is an oncogene and aberrant activation of its gene product due to missense mutations is seen in sporadic tumors. Similarly, increased activation of the abnormal gene product occurs due to the germline mutations in CS. While the vast majority of CS causing *HRAS* mutations occurs in the paternal germline, exceptions include two maternally derived germline mutations, one instance of somatic mosaicism resulting in an atypical presentation of CS, and one transmission from a parent with somatic mosaicism to a son with CS. A review of 139 individuals shows p.G12S to be the most common (113/139, or 81%), followed by p.G12A, seen in 7%. Other mutations were seen in fewer than four individuals each. The common mutations result in the typical phenotype, whereas the presentation of presumably more strongly activating mutations (p.G12V) appears to be more severe, and other changes (p.T58I) may be associated with a milder phenotype. Delineation of the full phenotypic spectrum associated with germline *HRAS* mutations is ongoing and may provide a baseline for future drug therapies directed at the hyperactive Ras/MAPK signaling pathway.

Cardio-Facio-Cutaneous Syndrome

Giovanni Neri. CFC is an autosomal dominant, multiple congenital anomalies/mental retardation (MCA/MR) syndrome, characterized by typical facies, ectodermal and cardiac involvement, and developmental delay. The main manifestations are relative macrocephaly, a broad forehead, downslanting palpebral fissures, a short nose with depressed root and bulbous tip, sparse and curly hair, sparse/absent eyebrows, cutaneous hyperkeratosis and hemangiomas, congenital heart defects (mainly pulmonic stenosis and hypertrophic cardiomyopathy), failure to thrive (at least in part due to severe feeding difficulties), epilepsy, and mental retardation. The publication of the initial cases and of a number of subsequent reports sparked a lively controversy over whether or not CFCS was distinguishable from the NS. Although a number of phenotypic manifestations do in fact overlap, others, such as neurocognitive delay and major ectodermal involvement, are more typical, if not exclusive, of CFCS. The controversy came to an end when genes responsible for the now called neuro-cardio-facio-cutaneous syndromes (NCFCS) were finally discovered. This family of syndromes, also including the CS, was found to be genetically heterogeneous, with several genes involved, encoding proteins acting in the Ras/MAPK pathway that regulates cell proliferation, differentiation, and death. The genes causing the majority of cases of CFCS are *BRAF*, *MEK1*, *MEK2*, and *KRAS*. These recent findings explain the phenotypic similarities and differences observed among the various forms of NCFCS, allow for more accurate genotype–phenotype correlations, and provide a detailed knowledge of the molecular mechanisms at the basis of these conditions, opening the way to possible pharmacologic treatments.

Phenotypic Overlap and Molecular Advances: What to Call a Syndrome in the Modern Era

John C. Carey. The recent advances in the identification of genes causing human congenital malformations and their syndromes have had a remarkable effect on our thinking about the definition and delineation of these entities. In many circumstances, we have discovered that a single gene is the molecular and causal basis for several previously recognized discrete syndrome phenotypes (e.g., *COL2* is the causative gene for classical Stickler syndrome, hypochondrogenesis, Kniest dysplasia, etc.), while a single well-known syndrome (e.g., Bardet–Biedel syndrome) can be due to 13 genes on different chromosomes. These advances have led clinical geneticists to wonder how we should refer to a condition, that is, by its traditional phenotypic name or according to its molecular basis. There are many examples of this scenario: The overlap of phenotypic signs among Stickler syndrome, Marshall syndrome, and the OSMED disorders leading to the type 1, type 2, type 3 Stickler classification; the "continuum" of Smith-Lemli-Opitz syndrome from a lethal phenotype (sometimes referred to as type 2 SLO) to a phenotype with only minor anomalies and developmental delays; and, of course, the phenotypic overlap among the community of Ras pathway conditions (CFC, CS, NS, NF1, NFNS, and Legius). Two recent papers have attempted to make sense of this apparent dilemma: (1) The Invited Editorial by Hennekam [2007] in *AJMG* led to his Patient Central proposal; (2) Robin and Biesecker [2001] proposed an axis system similar to what is used in the field of psychiatry. The speaker will address these themes in this presentation. The discussion will include the history of the concept of a syndrome, highlighting the international workshops in the 1980s, the axis system, and the provision of several examples illustrating the above concepts. The speaker recommends: (1) International consensus leading to publication of well-defined diagnostic criteria for the phenotypes of these syndromes; (2) Wider application of the axis system proposed by Robin and Biesecker; (3) Inclusion of the concepts of locus and allelic heterogeneity in clinical contexts.

Biochemistry and Function of Neurofibromin and SPRED1

Eric Legius. Neurofibromin is the first protein of the Ras/MAPK pathway that was identified to be mutated in a human heritable disorder. NF1 is caused by heterozygous mutations in the *NF1* gene localized at chromosome band 17q11.2 and coding for neurofibromin. NF1 is characterized by multiple café-au-lait spots, neurofibromas, Lisch nodules, learning difficulties and an increased risk of brain tumors and malignant peripheral nerve sheath tumors. Neurofibromin contains a central region with a GTPase activating protein (GAP) activity. The GAP-related domain of neurofibromin

(*NF1*GRD) stimulates the intrinsic GTPase function of the RAS proteins. Guanine exchange factors such as SOS1 will promote the activation of RAS proteins by stimulation of the transition of RAS bound to GDP (RAS-GDP) to RAS bound to GTP (RAS-GTP). Activated RAS (RAS-GTP) can stimulate the MAPK pathway by activating the RAF protein kinases. RAS-GTP can also activate the PI3-kinase pathway, the RAL-GDS pathway and PLCε. RAS-GTP is inactivated by GAP-proteins such as neurofibromin and p120-GAP, the protein coded by *RASA1*. Heterozygous mutations in *RASA1* cause the capillary malformation—arteriovenous malformation syndrome. Both *NF1* and *RASA1* are tumor suppressor genes. In NF1-associated tumors, the normal allele of the *NF1* gene is inactivated and the complete absence of neurofibromin in tumor cells results in activation of the RAS proteins. The RAS proteins remain in the GTP-bound state for a much longer period of time and this results in hyperactivation of their downstream targets. *NF1* mutations identified in individuals with NF1 are therefore inactivating mutations, also the mutations observed in the normal *NF1* allele in NF1-associated tumor cells are inactivating mutations. A heterozygous mutation in the *NF1* gene is responsible for the hyperactivation of the Ras/MAPK pathway in the neurons of the brain and this results in neurocognitive abnormalities. This has been studied extensively in the mouse and Drosophila models of Nf1. Neurofibromin deficiency in Drosophila results in a size defect that can be rescued by activation of the cAMP pathway. The size defect in Drosophila is also rescued by restoring the *NF1*GRD in Drosophila Ras2 neurons (Ras2 is the Drosophila ortholog of mammalian R-Ras). SPRED1 is a member of the SPROUTY/SPRED family of proteins which are negative regulators of Ras–Raf interaction and mitogen activated protein kinase (MAPK) signaling. We reported a NF1-like syndrome (Legius syndrome) in patients with a heterozygous inactivating mutation in the *SPRED1* gene. A biallelic mutation in *SPRED1* was demonstrated in a café-au-lait spot derived from melanocytes from a person with Legius syndrome. Legius syndrome is characterized by the same café-au-lait spots and freckling as in NF1 but no typical NF1-associated tumors are seen in Legius syndrome. The inactivating mutations in *SPRED1* result in an overactivation of the RAF kinases by activated RAS due to a reduction of the inhibitory effects of SPRED1 on the Ras–Raf interaction. It has been shown that Ras-GTP recruits Raf to the cell membrane and that the Ras–Raf interaction is associated with a phosphatase holoenzyme comprised of Shoc2/Sur8 and the catalytic subunit of PP1. It is believed that dephosphorylation of the S259 inhibitory site of Raf proteins by the Shoc2/Sur8-PP1c holoenzyme is necessary for Raf activation.

Biochemical and Functional Properties of SHP2

Marco Tartaglia. SHP2 is a cytoplasmic Src-homology 2 (SH2) domain-containing protein tyrosine phosphatase, functioning as a signal transducer downstream of growth factor and cytokine receptors. SHP2 positively controls signal flow through RAS, and is required during development. Germline missense mutations in *PTPN11*, the gene coding SHP2, have been discovered as major molecular events underlying the clinically related NS and LEOPARD syndrome, two developmental disorders with overlapping pleiomorphic phenotypes. A distinct class of missense mutations in the same gene has been identified as the most frequent somatic event in juvenile myelomonocytic leukemia, and occur with variable prevalence in childhood and adult hematologic malignancies and solid tumors. Available records based on more than 800 germline and somatic defects indicate that NS-causing and leukemia-associated *PTPN11* mutations are almost always missense changes, and are not randomly distributed throughout the gene. Biochemical and functional characterization of disease-associated mutations support the view that they can perturb SHP2 function by multiple mechanisms. Most lesions affect residues involved in the N-SH2/PTP interdomain binding network that stabilizes SHP2 in its catalytically inactive conformation or are in close spatial proximity to them. These mutations up-regulate SHP2 function by impairing the switch between the active and inactive conformation, favoring a shift in the equilibrium toward the latter, without altering SHP2's catalytic capability. A number of mutations, however, affect residues contributing to the stability of the catalytically inactive conformation but also participating in catalysis or controlling substrate specificity. For some of these defects, the individual substitution does not markedly perturb substrate affinity and/or catalysis, but rather protein activation by N-SH2 dissociation prevails. A few missense mutations affect residues located in the phosphopeptide binding cleft of each SH2 domain. Experimental evidence supports the idea that these amino acid substitutions promote SHP2 gain of function by increasing the binding affinity or altering the binding specificity of the protein for the phosphorylated signaling partners. LEOPARD syndrome-causing *PTPN11* mutations constitute a functionally distinct mutation group. While an impaired catalytic activity has been established as biochemical behavior shared by these SHP2 mutants, they do not appear to perturb intracellular signaling by a merely dominant negative effect, as supposed in the past.

Biochemical and Functional Characterization of Germline *RAS* mutations Associated With Ras/MAPK Pathway Syndromes

Suzanne Schubbert. Germline missense mutations in *HRAS* and *KRAS* and in genes encoding molecules that function up or downstream of Ras in cellular signaling networks cause a group of related developmental disorders that includes CS, NS, and CFC. Biochemical and functional studies show that syndrome-associated mutant Ras proteins demonstrate various abnormal biochemical properties and a range of gain-of-function effects in different cell types. Ras proteins are signal switch molecules that regulate cell fates by coupling receptor activation to downstream effector pathways to control diverse cellular processes including proliferation, differentiation, and survival. Ras proteins cycle between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound conformations (Ras-GTP and Ras-GDP). The competing activities of guanosine nucleotide exchange factors (GNEFs) and GTPase activating proteins (GAPs) regulate Ras-GTP levels. Activated growth factor receptors recruit signal relay proteins that stimulate GNEFs, which displace guanine nucleotides from Ras and permit passive binding to GTP. Ras-GTP interacts

productively with Raf-1, phosphatidylinositol 3-kinase, Ral-GDS, and other effectors. The intrinsic Ras GTPase terminates signaling by hydrolyzing Ras-GTP to Ras-GDP. This slow "off" reaction is greatly augmented by GAPs. Somatic missense *RAS* mutations that introduce amino acid substitutions at positions 12, 13, and 61 are frequently found in human cancers. Germline *KRAS* mutations that encode novel amino acid substitutions not found in cancer have been discovered in 2–4% of individuals with NS as well as in some persons with CFC. Studies show that syndrome-associated K-Ras proteins have distinct biochemical properties and are gain-of-function mutants that are less activated than oncogenic Ras proteins. Recombinant V14I and T58I K-Ras proteins display defective intrinsic GTP hydrolysis and impaired responsiveness to GAPs. P34R and D153V K-Ras show normal levels of intrinsic GTP hydrolysis, however F156L K-Ras is highly impaired. P34R K-Ras is insensitive to GAPs, and F156L K-Ras shows a weak response. Interestingly, F156L K-Ras displays a markedly increased rate of GTP and GDP dissociation, which is similar to an H-Ras mutant with a substitution found in CS (K117R). In addition, COS-7 monkey kidney cells expressing V14I, P34R, and F156L K-Ras show elevated levels of Ras-GTP that correlate with levels of phospho-MEK and phospho-ERK. V14I, T58I, P34R, D153V, and F156L K-Ras render primary myeloid and erythroid hematopoietic progenitors hypersensitive to growth factors, although at varied potency. The T58I substitution was identified in an infant with both NS and juvenile myelomonocytic leukemia, and interestingly primary macrophage progenitor cells expressing T58I K-Ras show markedly increased levels of Ras-GTP, phospho-MEK, and phospho-AKT. In summary, biochemical and functional studies of syndrome-associated Ras proteins supports the idea that the intrinsic Ras GTPase activity, the responsiveness of these proteins to GAPs, and guanine nucleotide dissociation all regulate developmental programs in vivo. Full author list: Suzanne Schubbert, Gideon Bollag, Christian P. Kratz, Martin Zenker, Charlotte M. Niemeyer, Ellen Daneyer, Eric Legius, Anders Molven, and Kevin Shannon.

Gain and Loss of Function Mutants Affecting the Ras/MAPK Pathway Can Cause Noonan Syndrome: *SOS1* and *RAF1*

Amy E. Roberts. All of the genes that are implicated in NS encode proteins that are components of the Ras/mitogen-activated protein kinase (MAPK) signal transduction pathway. After mutations in *PTPN11* were identified in 50% of cases of NS, a flurry of studies to identify other NS genes ensued. Using a candidate gene approach, it was shown that mutations in *SOS1* and *RAF1* cause 10%, and 3–17% of cases of NS, respectively. To date, no features have been found to exclusively correlate with a particular genotype though there are some genotype phenotype trends. Mutations in the genes implicated in NS typically activate the pathway, although often to a lesser extent than observed by somatic changes. However, exceptions to the gain-of-function mechanism of disease pathogenesis have been described. The effect of *SOS1* and *RAF1* mutations on the pathway and RAS and ERK activation will be discussed and the evidence that loss *or* gain of function mutants can act as a mechanism of NS clinical features will be reviewed.

Biochemical and Functional Characterization of BRAF and MEK in CFC Syndrome

Katherine A. Rauen. CFC is a rare multiple congenital anomaly disorder in which individuals have characteristic dysmorphic craniofacial features, cardiac defects, ectodermal anomalies, developmental delay and hypotonia. CFC is caused by alteration of activity through the mitogen-activated protein kinase (MAPK) pathway due to heterozygous germline mutations in protein kinases BRAF, MEK1, or MEK2. Mutations in *KRAS*, a small GTPase, have also been implicated as causing both CFC and NS; however, its role has yet to be well defined. The majority of germline mutations that cause CFC occur in *BRAF*. Unlike the mutation spectrum seen in cancer, the *BRAF* mutations in CFC individuals are more widely distributed affecting exon 6 and exons 11–16. The most common *BRAF* mutations occur in exon 6 and result in the missense substitution Q257R, in exon 12 at amino acid position E501 and in exon 11 consisting of the missense substitution G469E. Functional in vitro studies of these novel CFC mutant proteins demonstrate that BRAF may be kinase activated or kinase impaired. Missense mutations in *MEK1* and *MEK2*, which encode downstream effectors of BRAF, also cause CFC syndrome. MEK1 and MEK2 are threonine/tyrosine kinases with both isoforms having the ability to activate ERK1 and ERK2. *MEK1* and *MEK2* mutations comprise ~25% of mutations in CFC individuals in which a gene mutation has been identified. Mutations in *MEK1* and *MEK2* are seen in roughly equal frequency. The vast majority are missense substitutions located in exons 2 and 3. Rare in-frame deletions have also been identified. Functional studies of the proteins encoded by the novel CFC *MEK1/2* mutations have shown that all of the CFC mutant proteins studied are more active than wild-type MEK in stimulating ERK phosphorylation.

Preclinical Testing in Genetically Engineered Mouse Models

Kevin Shannon. Translating our molecular genetic understanding of human disease into new treatments has proven challenging. This is due, in part, to the fact that therapeutics is ultimately a biochemical problem that involves many issues that academic investigators are unfamiliar with such as developing and modifying small molecules; drug solubility, pharmacokinetics, and off-target effects; and realistically assessing the risks of a new treatment to patients versus the potential benefits. Treating human developmental disorders is further complicated by the young age of the patients who are most likely to benefit and by difficulties in defining objective endpoint for assessing clinical efficacy. The discovery that NF1 is caused by mutations in a gene that negatively regulates Ras-GTP levels established hyperactive Ras signaling as the cause of a human developmental disorder. It took over a decade to realize that NF1 was the "tip of the iceberg," and germline mutations in *PTPN11*, *SOS*, *HRAS*, *KRAS*, *BRAF*, and *MEK1* are now known to underlie a constellation of disorders that share some common features. Together, these genetic data infer that developmental programs are tightly regulated by activation of the canonical Ras/Raf/MEK/ERK pathway. The high incidence of somatic *RAS* gene mutations and inherent difficulties in directly reversing the adverse biochemical

properties of these mutant proteins has stimulated intense interest in developing anti-cancer agents that inhibit downstream kinases including Raf and MEK. While some of these drugs may also prove useful for treating developmental disorders, it is important to recognize that the goals of cancer therapy (killing cells with a specific somatic mutation) are fundamentally different from the goals of treating the complications of a developmental disorder (long term modulation of biochemical activity in dynamic populations of normal cells). Genetically engineered mouse models of human disease are providing mechanistic insights into disease causation and are also being harnessed to test rational new treatments for cancer and other disorders. An effort that is of interest is the Preclinical Trials Consortium, which is funded by the Children's Tumor Foundation to test targeted agents in accurate mouse models of tumors that arise in persons with NF1 and NF2. I will discuss the structure and early experience of this group, as this may prove useful for discussing how to implement preclinical trials in related developmental disorders.

Mouse Models of Neurofibromatosis-1 Optic Gliomas: From Cage to Clinic

David H. Gutmann. NF1 is the most common inherited cancer syndrome in which affected individuals develop low-grade gliomas. As such, NF1 provides a unique platform to understand the molecular and cellular underpinnings of these frequent pediatric brain tumors. Over the past 10 years, my laboratory has employed *Nf1* genetically engineered mouse (GEM) models to define the critical signaling pathways important for glioma cell growth, the contribution of the tumor microenvironment to gliomagenesis, the role of neural stem cells relevant to gliogenesis, and the impact of glioma formation/growth on the normal brain. Initial studies on the function of the *NF1* gene product, neurofibromin, demonstrated that it primarily functions as a negative regulator of the RAS proto-oncogene. To understand how neurofibromin regulates RAS activity relevant to glial cell growth and gliomagenesis, we examined the ability of neurofibromin to inhibit the activity of the three major RAS isoforms (Ha-RAS, K-RAS, and N-RAS) expressed in astrocytes. Surprisingly, *Nf1* loss in primary mouse astrocytes caused hyperactivation of K-RAS only, with no effects on the other RAS isoforms. In addition, genetic K-RAS inhibition alone was sufficient to reverse the abnormal proliferation and motility phenotypes observed in *Nf1*−/− astrocytes. Moreover, only K-RAS expression in astrocytes resulted in optic glioma formation in vivo. These findings demonstrate that K-RAS is the primary RAS isoform responsible for regulating *Nf1*−/− astrocyte growth and motility: The importance of K-RAS to gliomagenesis was further underscored by our detection of oncogenic K-RAS mutations in sporadic pediatric low-grade gliomas. Next, we sought to determine how K-RAS regulates astrocyte cell growth. Using an unbiased proteomics approach, we identified the mammalian target of rapamycin (mTOR) signaling pathway as a downstream effector of K-RAS activation in *Nf1*−/− astrocytes. We further showed that neurofibromin regulates astrocyte cell growth and motility in a RAS-, Akt-, and mTOR-dependent fashion in vitro and in vivo, and that mTOR signals through Rac1 to control these biological processes. The importance of mTOR activation to *Nf1* glioma growth was validated in preclinical studies, which demonstrated that rapamycin inhibition of mTOR suppressed optic glioma growth in our *Nf1* GEM model of optic glioma in a dose-dependent fashion. In an effort to develop a GEM model of *Nf1* optic glioma, we first conditionally inactivated the *Nf1* gene in glial fibrillary acidic protein (GFAP)-expressing glial cells in vivo. While these mice had increased numbers of proliferating glial cells in their brains, optic gliomas did not develop. To recapitulate the genetics of individuals with NF1, *Nf1*+/− mice with neurofibromin loss in glial cells were developed: Nearly 100% of these mice developed low-grade glial neoplasms involving the prechiasmatic optic nerves and chiasm by 3 months of age. These observations established an obligatory role for non-neoplastic *Nf1*+/− stromal cells in tumorigenesis. We identified *Nf1*+/− microglia in the developing tumor by 6 weeks of age. Subsequent experiments demonstrated that these *Nf1*+/− microglia elaborated key stromal signals (hyaluronidases and chemokines), which uniquely promoted the proliferation and survival of *Nf1*−/− astrocytes. Using both genetic and pharmacologic approaches, optic glioma growth could be suppressed by blocking *Nf1*+/− microglia function in vivo. Current studies are aimed at using *Nf1* GEM models to understand how microenvironmental cells and signals control the spatial and temporal pattern of gliomagenesis in children, and to design stromal-directed adjuvant therapies. Finally, the availability of GEM models of NF1-associated optic glioma lays the foundation for preclinical studies aimed at improving clinical outcome in children with these tumors. In addition to employing *Nf1* GEM optic glioma models to identify novel targets for chemotherapy and for preclinical drug evaluation, we have sought to determine the impact of glioma formation/growth on normal brain function. In the case of NF1-associated optic glioma, our current chemotherapy is reasonably effective at halting tumor growth; however, few children ever regain vision following treatment. To explore the basis for visual loss, we employed a combination of visual physiology, electron microscopy, and magnetic resonance imaging to demonstrate that optic glioma growth is associated with a time-dependent loss of axonal integrity and retinal ganglion cell death. Current studies are focused on identifying the signaling pathway responsible for *Nf1*+/− retinal ganglion cell survival and developing neuroprotective strategies as adjuvant approaches to brain tumor treatment.

Modeling the Effects of Oncogenic *BRAF* in Genetically Engineered Mice

Martin McMahon. Mutation of the *BRAF* proto-oncogene is detected in ~7% of all human malignancies and in the rare CFC syndrome. Most (but not all) of such mutations lead to constitutive activation of the BRAF → MEK → ERK MAP kinase pathway. The most common mutation detected in cancer, $BRAF^{T1799A}$, leads to expression of $BRAF^{V600E}$, which is a highly activated form of the protein. To model the effects of oncogenic $BRAF^{V600E}$, we used gene targeting in ES cells to generate mice carrying a Cre-activated allele of *BRaf* ($BRaf^{CA}$). Prior to Cre-mediated recombination, $BRaf^{CA}$ expresses normal *BRaf* as a fusion transcript between mouse exons 1–14 and human exons 15–18. However, following Cre-mediated recombination $BRaf^{CA}$ is rearranged to express $BRaf^{V600E}$ at normal levels of express under the control of the