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Disorders of dysregulated signal traffic through the RAS-MAPK pathway: phenotypic spectrum and molecular mechanisms

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Abstract

RAS GTPases control a major signaling network implicated in several cellular functions, including cell fate determination, proliferation, survival, differentiation, migration, and senescence. Within this network, signal flow through the RAF-MEK-ERK pathway, the first identified mitogen-associated protein kinase (MAPK) cascade, mediates early and late developmental processes controlling morphology determination, organogenesis, synaptic plasticity and growth. Signaling through the RAS-MAPK cascade is tightly controlled, and its enhanced activation represents a well-known event in oncogenesis. Unexpectedly, in the past few years, inherited dysregulation of this pathway has been recognized as the cause underlying a group of clinically related disorders sharing facial dysmorphism, cardiac defects, reduced postnatal growth, ectodermal anomalies, variable cognitive deficits and susceptibility to certain malignancies as major features. These disorders are caused by heterozygosity for mutations in genes encoding RAS proteins, regulators of RAS function, modulators of RAS interaction with effectors or downstream signal transducers. Here, we provide an overview of the phenotypic spectrum associated with germline mutations perturbing RAS-MAPK signaling, the unpredicted molecular mechanisms converging towards the dysregulation of this signaling cascade, and major genotype-phenotype correlations.

Keywords

RAS signaling; Noonan syndrome; LEOPARD syndrome; cardio-facio-cutaneous syndrome; Costello syndrome; neurofibromatosis type 1; Legius syndrome; Noonan-like syndrome with loose anagen hair

Introduction

RAS proteins are small guanosine triphosphate (GTP)/guanosine diphosphate (GDP)-binding GTPases that function as molecular switches controlling a major intracellular signaling network that, depending on the cellular context, guides diverse biological functions such as proliferation, migration, survival, cell fate determination, differentiation and senescence.[1,2] RAS signaling is switched on upon stimulation by numerous cytokines, hormones and growth factors, and mediates the appropriate cell response to external stimuli through the regulation of transcription, cytoskeletal rearrangement and metabolism. Within

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this network, signal flow through the RAF-MEK-ERK protein kinase pathway (Figure 1), the first described mitogen-associated protein kinase (MAPK) cascade, controls early and late developmental processes, including determination of morphology, organogenesis, synaptic plasticity and growth.[3,4] Signal transmission via this cascade is initiated by the activation of cell surface receptors by extracellular ligands, which creates intracellular docking sites for adaptor molecules and signal-relay proteins recruiting and activating guanine nucleotide-exchange factors (GEFs). In turn, GEF proteins promote the release of GDP from RAS, favoring binding to the more prevalent GTP and activation of the GTPase. Activated GTP-bound RAS interacts with the RAF serine/threonine kinases (RAF1, BRAF and ARAF) favoring their catalytic activation, which results in the phosphorylation and activation of their substrates, the MAPK/ERK kinases (MEK1 and MEK2). Upon activation, MEKs act as dual specificity kinases to phosphorylate regulatory residues of the extracellular signal-regulated kinases (ERK1 and ERK2), which function as serine/threonine proteine kinases to modulate the activity of a large number of cytoplasmic and nuclear substrates, including proteins implicated in the control of gene expression. Several feedback mechanisms negatively control signal flow through the RAS-MAPK pathway, including the functional inactivation of RAS by GTPase activating proteins (GAPs), which stimulate their intrinsically low GTPase activity.

Due to RAS's nodal role in signal transduction and cell function, signal traffic through it is tightly controlled. Loss of that control leading to enhanced flow contributes significantly to oncogenesis.[5–7] Activating somatic *RAS* gene mutations occur in approximately 30% of human cancers, but the upregulation of this signaling pathway can also result from enhanced function of upstream signal transducers or RAS effectors, as well as from inefficient function of feedback mechanisms. The recent, unpredicted discovery of germline mutations in a number of genes coding for proteins participating in RAS signaling, however, has established a novel scenario in which aberrant signal flow through RAS is causally linked to a group of clinically related developmental disorders characterized by facial dysmorphism, a wide spectrum of cardiac disease, reduced growth, variable cognitive deficits, ectodermal and musculoskeletal anomalies, and increased risk for certain malignancies (Figure 1).[8,9] This group of diseases can now be viewed as a family of disorders of upregulated RAS-MAPK signaling. Based on the shared pathogenic mechanism and clinical overlap, these genetic diseases have been tentatively grouped as neuro-cardio-facial-cutaneous syndromes (NCFCS) or RAS-opathies.[10,11]

In this review, we outline the phenotypic spectrum associated with perturbed RAS-MAPK signaling, the molecular mechanisms converging towards the dysregulation of this pathway, and major genotype-phenotype correlations.

The RAS-opathies

Noonan syndrome

Noonan syndrome (NS, OMIM 163950) is a disorder characterized by particular facial dysmorphic features, congenital heart defects (CHD), hypertrophic cardiomyopathy (HCM), short stature, skeletal anomalies, and webbing of the neck. Other relatively common features are bleeding diathesis, ectodermal anomalies, lymphatic dysplasias, cryptorchidism, and cognitive deficits.[12–14] NS is characterized by marked variability, which is, in part, explained by the underlying molecular lesion (see below). Facial features and signs include high forehead, hypertelorism, downslanting palpebral fissures, epicantal folds, ptosis, low-set and/or posteriorly rotated ears (Figure 2). Besides the short and/or webbed neck, a low posterior hairline commonly occurs. Affected individuals exhibit a wide spectrum of cardiac disease.[15,16] Pulmonic stenosis (PS) and HCM occur most commonly, but other lesions are also observed. Feeding problems are noted in the majority of affected infants and can cause

failure to thrive.[17] While feeding difficulties resolve in most patients by around age 18 months, the inadequacy in oral intake may be severe enough to necessitate placement of gastrostomy tubes in some.

Developmental delay and learning problems are quite common. Some motor delay can be attributed to the hypotonia often observed in affected infants. An increased prevalence of attention deficit/hyperactivity disorder and frank mental retardation are also observed. Available data indicate that the heterogeneity in cognitive abilities observed in NS is at least partially ascribed to the specifics of the causative gene mutation.[18,19] Skeletal anomalies most frequently consist of pectus deformities, cubitus valgus, and vertebral defects. Of note, two childhood leukemias, juvenile myelomonocytic leukemia (JMML, OMIM 607785) and acute lymphoblastic leukemia (ALL), arise at increased prevalence in NS although they affect only a small percentage of patients.[20–22] The myeloproliferative disorder in children with NS (NS/JMML) may regress without treatment, follow an aggressive clinical course or evolve to acute myeloid leukemia. This condition is strictly associated with a narrow spectrum of mutations affecting the *PTPN11* gene (see below).[23,24]

NS is thought to be relatively common, although its prevalence has not been determined accurately to date. Most authors cite the figure of 1 in 1,000–2,500 live births, but that estimate is not based on a population study.[25] NS is a Mendelian trait transmitted in an autosomal dominant manner, and, typical for that genetic mechanism, 1/3 to ½ of cases arise from spontaneous mutations. Thus far, seven genes (*PTPN11*, *SOS1*, *KRAS*, *NRAS*, *RAF1*, *BRAF* and *MEK1*) have been causally related to this disorder. Mutations in these genes account for a large percentage of cases (approximately 70–75%, in our experience), but is highly dependent on the diagnostic criteria used to make the diagnosis. A recent review provides an up-to-date and thorough survey of the clinical issues and pathogenetic mechanisms for this disorder.[26]

LEOPARD syndrome

LEOPARD syndrome (LS, OMIM 151100) is an autosomal dominant trait that overlaps phenotypically with NS. It is also allelic with NS, with a restricted spectrum of mutations in *PTPN11* accounting for the vast majority of affected individuals (see below).[27,28] In a small proportion of cases, LS has been causally linked to mutations in *RAF1* or *BRAF*.[29–31] The acronymic name refers to the major features: Lentigines, ECG conduction abnormalities, Ocular hypertelorism, Pulmonic stenosis, Abnormal genitalia, Retardation of growth, and sensorineural Deafness.[32] Similar to NS, there are age-dependent aspects of the phenotype. Facial dysmorphism is similar to that of NS but is usually milder,[33] and the neck is short but not webbed. Multiple lentigines present as dispersed flat, black-brown macules, mostlyon the face, neck, and upper part of the trunk, but sparing the mucosae. In general, lentigines appear at the age of 4–5 years and increase until puberty into the thousands. Café-au-lait spots are also observed, alone or in association withlentigines, in up to 70–80% of the patients,[33] and usually precede the appearance of lentigines, being present from the first months of life. Growth retardation (height below the 3rd centile) is observed in 25% of affected individuals, and final height in 85% of subjects is below the 25th centile.[34,35] Approximately half of affected individuals have heart defects, which are similar to those recurring in NS but recur with different frequencies. ECG anomalies, progressive conduction defects and HCM are the most frequent features. Of note, HCM is detected in up to 80% of LS patients with heart defects, most commonly appearing during infancy. It is progressive and paralleled by the appearance of lentigines.[36] Diagnostic criteria for LS have been outlined by Voron and colleagues.[34] A clinical diagnosis is not always feasible, particularly in young patients with no lentigines, due to the overlap with NS and neurofibromatosis-Noonan syndrome (see below). In these patients, the clinical differentiation from NS may be difficult during infancy and early childhood. The occurrence

of distinct associated signs, including HCM, sensorineural deafness, and café-au-lait spots represents an important diagnostic handle in these young patients.[33] Sarkozy *et al*.[37] provide an updated review of the clinical and molecular genetics aspects of this disorder.

Noonan syndrome-like with loose anagen hair

Subjects with Noonan syndrome-like with loose anagen hair (NS/LAH; OMIM 607721) show features that are at first view reminiscent of NS (Figure 2). The phenotype of these subjects, however, is notable for reduced growth associated with proven GH deficiency, cognitive deficits, distinctive hyperactive behaviour, and easily pluckable, sparse, thin, slow growing hair in the anagen phase but lacking an inner and outer root sheaths.[38,39] Such hair anomalies fit a well-known condition termed loose anagen hair (LAH) syndrome, which is clinically heterogeneous and has been reported to occur typically in young children and, in most cases, spontaneously improves clinically as early as adolescence.[40] In these subjects, LAH can be confirmed by microscopic examination of plucked hairs.[41] Most affected individuals exhibit hairless and darkly pigmented skin with eczema or ichthyosis, and a tendency to pruritus. Ectodermal anomalies also include sparse eyebrows and dystrophic or thin nails. The voice is characteristically hoarse or hypernasal. Cardiac anomalies are observed in the majority of cases, with dysplasia of the mitral valve and septal defects significantly overrepresented compared with the general NS population. So far, NS/LAH appears to be genetically homogeneous, as all affected individuals share the same 4A>G missense change (Ser2Gly) in *SHOC2*, which encodes a scaffold protein required for the efficient transmission of information from RAS to the MAPK cascade.[39]

Noonan-like/multiple giant cell lesion syndrome

NS is rarely accompanied by multiple giant cell lesions (MGCLs) of the jaw and/or other bone/soft tissues. This finding was originally introduced as a distinct nosologic entity named Noonan-like/multiple giant cell lesion syndrome (NL/MGCLS) or Noonan syndrome with cherubism (OMIM 163955).[42] Now, it is apparent that MGCLs are an associated feature of NS, LS and cardiofaciocutaneous syndrome and that NL/MGCLS is not a separate disorder. This view is supported by the observation that this feature has been documented to occur in different conditions and be caused by mutations affecting *PTPN11*, *SOS1* and *MEK1* genes (see below) previously reported in subjects with those syndromes without any evidence of bony involvement.[43–50] Based on these considerations, the use of NL/MGCLS should be avoided.

Cardiofaciocutaneous syndrome

Cardiofaciocutaneous syndrome (CFCS, OMIM 115150) is a rare, sporadic multiple congenital anomalies/mental retardation syndrome characterized by failure to thrive, severe feeding problems, developmental delay, reduced growth, distinctive dysmorphic facial features, abnormalities of the skin, gastrointestinal tract and central nervous system, and cardiac defects.[51,52] CFCS has considerable clinical overlap with NS, and “borderline” cases are commonly observed, which justified the long debated question of whether it was a separate nosologic entity or an extreme phenotype/variant of NS.[53–55]

Recurrent facial/head features include relative/absolute macrocephaly, which is usually associated with high forehead, bitemporal narrowing and facial dysmorphia that is coarser compared to NS (Figure 2). The cutaneous involvement includes dry and hyperkeratotic skin (face, arms and legs), ichthyosis, eczema, sparse, friable and curly hair, absent/sparse eyebrows and eyelashes, pigmentary changes (such as café-au-lait spots, naevi or lentigines) and hemangiomata. Heart defects occur in the majority of affected individuals and consist of pulmonic stenosis most commonly, HCM and septal defects.[52] Cognitive deficits are usually moderate to severe. Hypotonia results in marked motor delay. Structural brain abnormalities

include cerebral atrophy, frontal lobe, corpus callosum or cerebellar vermis hypoplasia, and hydrocephalus.[52] Seizures are also frequently observed. As with Costello syndrome (see below), life expectancy is shortened on average due to the early death of affected individuals with severe cardiac involvement.

CFCS is genetically heterogeneous, with mutations in the *KRAS*, *BRAF*, *MEK1* and *MEK2* genes occurring in approximately 60–90% of affected individuals.[56–60]

Costello syndrome

Costello syndrome (CS; OMIM 218040) is the eponymous name for the disorder originally described in 1971, and further delineated by the same author in 1977, as a condition characterized by prenatal overgrowth followed by postnatal feeding difficulties and severe failure to thrive, distinctive coarse facial features (Figure 2), mental retardation, short stature, cardiac defects (most commonly hypertrophic cardiomyopathy, septal defects, valve thickening and/or dysplasia, and arrhythmias), and musculoskeletal and skin abnormalities.[61–64] Children and young adults with CS are predisposed to malignancies.[65] Solid tumors, including rhabdomyosarcoma and less frequently neuroblastoma and bladder carcinoma, have been estimated to occur in approximately 15% of affected individuals throughout childhood, while benign cutaneous papillomata in the perinasal or/and perianal region(s) represent a distinctive and common feature associated with the disorder.

CS is caused by germline missense mutations in the *HRAS* proto-oncogene (OMIM 190020).[66] Data from published surveys indicate that *HRAS* mutations account for 83% to 100% of subjects with a confirmed diagnosis of CS.[66–70] This finding has been explained as either locus heterogeneity or by the difficulties of diagnosis in cases exhibiting overlapping features with clinically related disorders or a phenotype that evolves with time. In our opinion, CS should be used only for those subjects who are heterozygous for a *HRAS* mutation. The absence of a mutation in the gene in a subject with phenotype apparently fitting CS would be predictive of a clinically related but nosologically distinct condition. Clinical re-evaluation of *HRAS* mutation-negative cases is often helpful diagnostically.

Neurofibromatosis type I, including Neurofibromatosis-Noonan syndrome and Watson syndrome

Neurofibromatosis type 1 (NF1; OMIM 162200) is one of the most common autosomal dominant diseases (1 in 3000–4000 live births). Its main clinical characteristics include cutaneous, subcutaneous and/or plexiform neurofibromas, café-au-lait spots, axillary and/or inguinal freckling, Lisch nodules in the iris, skeletal deformities, vascular defects, learning disabilities and behavioral problems, short stature, macrocephaly and a predisposition for developing benign and malignant neoplasias, most commonly myeloid malignancies, including JMML, optic gliomas, pheochromocytomas, rhabdomyosarcomas and neuroblastomas.[71,72] The diagnosis of NF1 is based on clinical criteria established at the NIH Consensus Development Conference in 1988.[73] It should be noted, however, that subjects diagnosed as having NF1 using those criteria may carry heterozygous mutations in *SPRED1*, which underlie Legius syndrome (see below). NF1 is caused by heterozygous loss-of-function mutations or deletions of the *NF1* gene, encoding the RAS-specific GTPase, neurofibromin.[74] Loss-of-function *NF1* mutations occur in the vast majority of affected individuas (> 90%). NF1 is essentially fully penetrant but there is considerable variation in the clinical features of the disease, even within families transmitting the trait. Fernel[75] and Lee and Stephenson[76] provide updated reviews of the clinical and molecular genetic aspects of this disorder.

Neurofibromatosis-Noonan syndrome (NFNS, OMIM 601321) is a clinical trait first noted in 1985 by Allanson and co-workers who described subjects with features of both NF1 and NS (Figure 2).[77] It has been long questioned whether NFNS was a variant of either NF1 or NS, a chance association, or a distinct disorder.[78,79] Recent reports have provided some insights but have not completely resolved that issue. *NF1* mutation analysis performed in three independent cohorts of patients with features fitting NFNS documented occurrence of heterozygous *NF1* mutations in 23 of 28 subjects.[80–82] Lesions included nonsense mutations, out-of-frame deletions, missense changes (mostly affecting the GAP domain), and small in-frame deletions. Those groups also searched for *PTPN11* mutations in their subjects but failed to find any. In contrast, two cases of NFNS with *NF1* and *PTPN11* mutations have been reported.[83,84] Finally, *NF1* mutations have co-segregated with the NFNS trait in a few kindreds large enough to make second mutations highly unlikely. Taken together, the current evidence indicates that most individuals with NFNS harbor an *NF1* mutation and that a single mutation appears to be sufficient to engender the trait. Double heterozygosity for *NF1* and *PTPN11* mutations rarely causes NFNS, and it is not yet clear how frequently mutations in other NS disease genes co-occur with *NF1* defects in the disorder. These findings support the view that NFNS is genetically distinct from NS and emphasize the extreme phenotypic variability associated with lesions in the *NF1* gene. The identification of specific *NF1* alleles recurring in NFNS, the fact that these alleles cosegregate in families with the condition, and the observation of a peculiar mutational spectrum strongly suggest that the term “NFNS” primarily characterizes a phenotypic variant of NF1, which manifests with a lower incidence of plexiform neurofibromas, skeletal anomalies, and internal tumors, in association with hypertelorism, ptosis, low-set ears, and CHDs.[79–82,85] It should be noted, however, that some of the mutations identified in patients with NFNS have also been reported in NF1 without any feature suggestive of NS.

A second trait closely related to NF1 is Watson syndrome (WS, OMIM 193520), an autosomal dominant condition reported by Watson in 1967,[86] characterized by pulmonic stenosis, café-au-lait spots, reduced growth and decreased intellectual ability.[86–89] This phenotype was originally considered distinct from NF1 and LS based on the absence of neurofibromata, Lisch nodules, lentigines, and deafness.[86,87] Allanson and co-workers, however, made observations expanding the clinical phenotype to include relative macrocephaly, Lisch nodules, and neurofibromata as recurrent features of the disorder.[88,89] Consistent with the clinical overlap with NF1, linkage to the *NF1* locus was documented,[89,90] and allelism between these two traits was proven by the identification of a 80-kb *NF1* deletion in a patient,[91] and an in-frame tandem duplication of 42 bases in exon 28 of the gene in an additional family.[92] The available records indicate that subjects with WS show only a mild expression of NF1 and lack the typical facial appearance of NS. As in the case of NFNS, WS should be considered as part of the broad clinical spectrum associated with *NF1* mutations.

Legius syndrome

Legius syndrome (OMIM 611431), previously termed neurofibromatosis type 1-like syndrome, is an autosomal dominant condition characterized by multiple axillary freckling, café-au-lait spots, macrocephaly, and a NS-like facial dysmorphism in some individuals.[93–96] Some patients have learning difficulties and/or hyperactive behavior. Despite the clinical overlap with NF1, affected individuals do not have Lisch nodules, cutaneous or plexiform neurofibromata, typical NF1 osseous lesions, or symptomatic optic gliomas, while lipomas represent a relatively common feature.[93,94,96] Additional studies are required, however, to establish susceptibility of affected individuals to malignancies.

Legius syndrome is caused by loss-of-function mutations of the *SPRED1* gene (see below).[93] Messiaen and co-workers observed that, among 42 *SPRED1* mutation-positive

individuals, approximately half of the cases fulfilled NIH's diagnostic criteria for NF1.[96] In the same study, mutational analysis of a large cohort of patients fulfilling NIH criteria for diagnosis of NF1 revealed a *SPRED1* mutation in 2% of cases (*i.e.*, approximately 8% of *NF1* mutation-negative cases).[96]

RAS-MAPK signaling dysregulation and phenotypic consequences

PTPN11 gene

The *PTPN11* gene (OMIM 176876) encodes SHP2, a widely expressed cytoplasmic protein tyrosine phosphatase that positively modulate RAS signaling.[97–100] It is characterized by two tandemly arranged *N*-terminal src-homology 2 domains (N-SH2 and C-SH2), a single catalytic domain (PTP) and a *C*-terminal tail of uncharacterized function. SHP2 binds to phosphotyrosyl-containing signaling partners through the SH2 domains, which control both SHP2's subcellular localization and function.[101]

We discovered *PTPN11* as a major NS disease gene using a positional candidacy approach in 2001.[102] *PTPN11* mutations account for approximately 50% of NS, and are more prevalent among subjects with pulmonary valve stenosis and short stature, and less common in individuals with HCM and/or severe cognitive deficits.[43,103] These mutations are almost always missense changes and perturb SHP2's function through distinct mechanisms.[104–106] Most mutations affect residues involved in the N-SH2/PTP interdomain binding network that stabilizes SHP2 in its catalytically inactive conformation (Figure 3). These mutations are predicted to up-regulate SHP2's function by impairing the switch between the active and inactive conformations, favoring a shift in the equilibrium toward the former. There are, however, changes affecting residues located in the phosphopeptide binding pocket of the N-SH2 and C-SH2 domains or in the linker stretch connecting these domains. There is evidence supporting the idea that these substitutions promote SHP2 gain of function by increasing binding affinity, altering the binding specificity for the signaling partners, or altering the flexibility of the N-SH2 domain in a manner that inhibits the N-SH2/PTP interaction.

A distinct class of *PTPN11* mutations has been identified to underlie LS.[27,28] Tyr279Cys and Thr468Met represent the most common defects, although additional mutations have been documented.[37] An impaired catalytic activity has been established as the biochemical behavior shared by these SHP2 mutants.[105–108] Their role in SHP2 functional dysregulation still requires further study, since they do not appear to perturb intracellular signaling by a merely dominant negative effect,[106,109,110] as supposed in the past.[108]

Children with NS are predisposed to a spectrum of hematologic abnormalities and malignancies, including JMML, a clonal myeloproliferative disorder of childhood,[111] and children with NS/JMML are heterozygous for *PTPN11* germline mutations in the majority of cases, with one mutation rarely observed in NS (218C>T, Thr73Ile) occuring in a large percentage of cases.[23,24,112] Remarkably, a distinct class of mutations in this gene, acquired as a somatic event, occurs in approximately one-third of children with JMML without NS as well as variable proportions of other myeloid and lymphoid malignancies of childhood.[23,24,112–115] As observed in NS, the vast majority of *PTPN11* lesions identified in this heterogeneous group of hematologic malignancies are missense changes that alter residues located at the interface between the N-SH2 and PTP domains (Figure 3), but appear to be more activating.[104,105,112,116,117]

De novo duplications of the chromosomal region encompassing *PTPN11* (12q24) have been documented in two subjects with clinical features suggestive of NS without mutations in any known NS genes.[118,119] These findings suggest that increased dosage of SHP2 may have

dysregulating effects on intracellular signaling and consequences on developmental processes. The screening of a relatively large of cohort of NS cases negative for mutations in previously identified genes, however, indicate that *PTPN11* gene duplication represents an uncommon cause of NS.

SOS1 gene

Cell-surface tyrosine kinase receptors activate RAS proteins by recruiting GEFs to the cytoplasmic side of the plasma membrane. These proteins catalyze the release of GDP from RAS, facilitating the conversion of the inactive GDP-bound form of RAS to its active GTP-bound state.[120] Among the GEFs, two members of the son of sevenless (SOS) family, SOS1 and SOS2, promote guanine nucleotide exchange on RAS proteins, but not on RAS-related family members, such as the RAP and RHO proteins.[121,122]

Based on their modulatory role in RAS signaling, we and others discovered that *SOS1* (OMIM 182530) is mutated in a relatively large percentage of subjects with NS.[123,124] This gene encodes a large multidomain protein (Figure 4).[125] The *N*-terminal portion of the protein contains a histone domain (HD) that is characterized by two tandemly arranged histone folds, and is followed by Dbl homology (DH) and pleckstrin homology (PH) domains, which are implicated in the activation of RAC, a small GTPase of the RHO/CDC42 family.[126] The *C*-terminal half of the protein contains the RAS exchanger motif (REM) domain and the Cdc25 domain, which are required for the RAS-specific nucleotide exchange activity of SOS1. Finally, the region at the *C*-terminus contains recognition sites for SH3 domains and mediates interaction of SOS1 with SH3 domain-containing adaptor proteins that deliver SOS1 to the membrane upon receptor activation. Additional anchorage sites on the membrane are provided by the phosphatidylinositol phosphate-binding site within the PH domain,[127] and an extended positively charged surface of the HD domain.[128]

The available data indicate that *SOS1* is the second most frequently mutated NS disease gene, accounting for approximately 10% of subjects.[123,124,129] The vast majority of the mutations identified are missense changes and affect multiple domains (Figure 4). Approximately half of the *SOS1* defects affect residues located in the short helical linker connecting the PH and REM domains, with substitutions of residue Arg552 accounting for approximately 30% of total mutations. A second mutation cluster is located within the PH domain (residues 432 to 434), while a third functional cluster resides at the interacting regions of the DH (Thr266 and Met269) and REM (Trp729 and Ile733) domains. A single amino acid change (Glu846Lys) within the Cdc25 domain accounts for more than 10% of defects.

Subjects heterozygous for a mutated *SOS1* allele tend to exhibit a distinctive phenotype characterized by ectodermal abnormalities generally associated with an absence of cognitive deficits and normal growth.[124,129] A few *SOS1* mutation-positive individuals with ectodermal manifestations and distinctive facial dysmorphia that might be suggestive of CFCS have been reported (Figure 2).[130,131] In these subjects, cognitive deficits were generally absent or minor.

The GEF activity of SOS1 is controlled by an allosteric site, involving the Cdc25 and REM domains, that stimulates SOS1 exchange activity through the binding of nucleotide-bound RAS.[132] Basally, the interaction between the DH and REM domains stabilizes SOS1 in its catalytically inactive conformation by masking the allosteric binding site for RAS. Following SOS1's translocation to the membrane, the inhibitory effect of the DH domain is relieved by still undefined events allowing RAS binding to the allosteric site, which in turn promotes a conformational change of the REM and Cdc25 domains, and RAS binding to the catalytic site.[133] Most NS-causing *SOS1* mutations reside in regions within the molecule that

are predicted to contribute structurally to the maintenance of the catalytically autoinhibited conformation (Figure 4). Biochemical data confirmed such predictions, demonstrating that NS-causing *SOS1* mutations promote gain of function and enhance RAS and ERK activation.[123,124]

A possible contribution of activating *SOS1* mutations in cancer has been evaluated (our unpublished data).[134,135] Mutational analysis performed on genomic DNA from more than 1,000 solid tumors (pancreatic, lung, breast, colon, thyroid cancers) and hematological malignancied (myeloproliferative disorders and acute myeloid and lymphoblastic leukemias) indicate that *SOS1* is not mutated in most cancers, suggesting that NS patients heterozygous for a *SOS1* mutation are not at increased risk of developing cancer.

HRAS, KRAS and NRAS genes

The three members of the RAS subfamily, HRAS (OMIM 190020), KRAS (OMIM 190070) and NRAS (OMIM 164790), are monomeric GTPases that use GDP/GTP-regulated molecular switching to control intracellular signal flow.[1,136] They exhibit high affinity for GDP and GTP, low GTPase activity, and cycle from a GDP-bound inactive state to a GTP-bound active state, the latter favoring their interaction with multiple downstream transducers. As anticipated, GDP/GTP cycling is controlled by GEFs, such as SOS1, which promote release of GDP, and GAPs, which accelerate the intrinsic GTPase activity. RAS proteins share a structure that includes a conserved domain (residues 1 to 165), known as the G domain, which is required for its signaling function, and a less conserved *C*-terminal tail, called the hypervariable region, that guides post-translational processing and plasma membrane anchoring (Figure 5). Within the G domain, conserved sequence elements direct GTP/GDP binding and GTP hydrolysis. Furthermore, two tracts, denoted as Switch I and Switch II, undergo major conformational changes upon GTP/GDP exchange and mediate binding to RAS effectors, GAPs and GEFs.[1,137] Heterozygous germline mutations in each of the three members of the RAS subfamily have been identified to be causally related with NS, CFCS or CS.

Based on the link between the functions of SHP2 and RAS, Aoki and co-workers used a candidate gene approach to discover that a small number of missense mutations affecting residues Gly12 and Gly13 in *HRAS* underlie CS.[66] These residues represent hot spots for oncogenic somatic mutations.[138] Defects affecting those residues render the protein insensitive to GAP-promoted GTP hydrolysis, resulting in a constitutively GTP-bound, active state.[5,138] Other less common amino acid substitutions involving residues in the purine ring binding pocket of the protein (His117 and Ala146) are predicted to affect the stability of HRAS binding to GDP/GTP,[69,70] favoring a shift in the equilibrium towards the GTP-bound, active form of RAS, which bypasses the requirement for a GEF.[139–142] Recently, two different three-nucleotide insertions resulting in the duplication of Glu37 within the switch I region have been reported.[143] This small in-frame insertion promotes a weak hyperactivation of signaling resulting from a balancing effect between a profound GAP insensitivity and inefficient binding to effector proteins.

In CS, the Gly12Ser substitution in HRAS accounts for approximately 85% of affected individuals. This change recurs with considerably lower prevalence in human cancers (approximately 5% of total HRAS amino acid substitutions) (COSMIC; http://www.sanger.ac.uk/genetics/CGP/cosmic/). Experimental data indicate that Gly12Ser has a lower transformation potential than the Gly12Val mutant,[144] which is the most common somatic HRAS defect in human tumors (about 45% of total somatic *HRAS* gene mutations). The latter has been documented in only two individuals with CS who exhibited a relatively severe phenotype (approximately 2% of cases with an *HRAS* gene mutation).[66,145] Similarly, no *HRAS* mutation affecting residue Gln61, which constitute approximately 33%

of total *HRAS* lesions in human cancer, has been documented in CS to date. Such a striking difference in prevalence and spectrum of HRAS amino acid substitutions suggests that CS-causative mutations have less potency for deregulating protein function than cancer-contributing ones and that the latter might exert a stronger perturbing effect on development when inherited.

Based on the identification of *HRAS* gene mutations in CS, we and others discovered that germline *KRAS* mutations can underlie NS or CFCS (Figure 2).[56,146,147] Mutations account for a relatively small percentage of affected subjects in both disorders (< 2–5%), and, in NS, are generally associated with a highly variable but generally severe phenotype. The diversity of mutations associated with these developmental disorders as well as their phenotypic spectrum have been investigated further, refining the picture of a clustered distribution of germline disease-associated *KRAS* defects, and confirming the high clinical variability.[59,148,149] The *KRAS* gene (OMIM 190070) produces two transcripts through alternative splicing, resulting in two proteins called KRASA and KRASB,[150] which differ at the *C*-terminus. Most *KRAS* mutations cluster in the first and second coding exons of the gene, which are shared by the two KRAS isoforms. Mutations affecting exon 6, however, have been documented in one-third of NS or CFCS subjects with a *KRAS* germline mutation. This exon codes for the *C*-terminal tail of KRASB but not KRASA. These findings document that isolated KRASB gain of function is sufficient for disease pathogenesis, which is also consistent with the available evidence about the relative roles of the KRAS isoforms on development from knock-out animal models.[151–153]

As observed for the somatically acquired oncogenic *RAS* gene mutations and germline transmitted *HRAS* lesions, the NS/CFCS-causing *KRAS* defects were found to up-regulate protein function by either affecting the intrinsic and/or GAP-stimulated GTPase activity and, consequently, impairing the switch between the active and inactive conformation, or determining a dramatically increased rate of guanine nucleotide dissociation.[147,154] Although these studies documented a complex pattern of intrinsic biochemical properties, expression of these mutants in COS-7 cells promoted higher levels of phosphorylated MEK and ERK proteins, indicating hyperactivation of the MAPK cascade. Of note, available records indicate that germline *KRAS* mutations are rarely observed in human cancers. This finding is consistent with functional data indicating a reduced perturbing effect of these mutations on protein function compared with the cancer-associated ones.[147,154] This is also supported by the observation that malignancies have been rarely observed in subjects with NS or CFCS due to a KRAS mutation.[56,59,147,148]

More recently, two germline missense mutations (Thr50Ile and Gly60Glu amino acid changes) have been reported to account for a few NS cases.[155] A germline *KRAS* mutation affecting Gly60 (Gly60Arg) has been reported in CFCS,[56] and the somatic *NRAS* mutation Gly60Glu rarely has been observed to occur in malignancies (COSMIC database). Of note, mutations of the neighbouring Gln61 are frequent oncogenic RAS alterations and confer impaired GTPase function. In contrast, no somatic or germline *RAS* gene mutation affecting Thr50 has been described so far. Thr50 is an exposed residue located in the β2-β3 loop connecting the two switch regions but is not predicted to be directly involved in GTP/GDP binding, GTP hydrolysis, or effector interaction. Functional characterization of these disease-associated mutations documented that, similarly to what was observed for *KRAS* and *HRAS* mutation, they confer enhanced stimulus-dependent MAPK activation but apparently through different molecular mechanisms.[155]

NF1 gene

The *NF1* gene (OMIM 16200) encodes neurofibromin, a cytoplasmic multifunction protein ubiquitously expressed with highest expression in neurons, Schwann cells,

oligodendrocytes, astrocytes and leukocytes.[156] The *NF1* coding sequence spans over 61 exons distributed over more than 300 Kb. A number of variant transcripts have been identified, which originate from alternative splicing, exon skipping and short sequence insertions. While neurofibromin is a large protein, it has no clearly defined functional domains, except the SEC14 and RasGAP domains, the latter mediating the RAS-specific GAP function of the protein. Besides its role as a negative modulator of RAS signaling, neurofibromin is involved in the modulation of other signaling pathways, including the protein kinase A (PKA)-cAMP pathway, and in microtubule-associated processes.[157–160] Moreover, a possible function within the nucleus has been hypothesized.[161] For a general overview see Trovó-Marqui and Tajara.[156]

In the majority of affected individuals, disease-causing *NF1* mutations consist of intragenic frameshift, nonsense, splicing or missense mutations arrayed along the entire length of the gene. A small proportion of affected individuals (< 5%) carry a heterozygous deletion of approximately 1.5 Mb, which is the result of unequal homologous recombination and encompasses the entire *NF1* and a few adjacent genes.[162–164] There is poor correlation between classes of mutations and disease features, with the exception of patients with large deletions who tend to have more severe phenotype and higher numbers of neurofibromata. Phenotypic heterogeneity is high among affected individuals, even within families co-inheriting the same *NF1* mutation.

NF1 is a tumor suppressor gene, and loss of the wild-type allele has been demonstrated to occur somatically as a second hit in malignancies associated with the trait.[165–167]

SPRED1 gene

The SPROUTY-related ENA/VASP homology-1 (EVH1) domain-containing protein 1 (SPRED1; OMIM 609291) is a member of the SPROUTY/SPRED family of membrane-associated proteins that function as modulators of growth factor–induced activation of the RAS-MAPK pathway. SPRED1 is characterized by an *N*-terminal EVH-1 domain, which binds to proline-rich sequences, a central KIT–binding domain (KBD) with unknown function, and a *C*-terminal SPRY domain implicated in protein translocation to the plasma membrane, RAF binding and heterodimerization with other SPRED proteins.[168] SPRED1 negatively controls MAPK signaling and operates downstream of RAS by suppressing RAF phosphorylation and activation,[169] although a target upstream of RAS has also been suggested.[170] Heterozygous loss-of-function mutations in *SPRED1* cause Legius syndrome.[93] The mutational spectrum is wide, including nonsense, frameshift and missense mutations, as well as small in-frame deletions.[93–96] Most mutated alleles produce unstable proteins that are likely to be quickly degraded in the cell, but others encode non-functional proteins. It has been demonstrated that disease-causing mutations resulted in increased RAF1 kinase activity, enhanced phosphorylation levels of MEK and ERK proteins, and increased activity of ELK1, a transcription factor functioning downstream the MAPK cascade.[93] Of note, as documented in NF1, second hits in the gene have been documented in melanocytes from café-au-lait spots of affected subjects, indicating that this feature is caused by biallelic inactivation of *SPRED1*, as previously demonstrated for NF1.[171] Mutational screening performed on kidney and cell nervous system cancers do not suggest a major contribution of somatic *SPRED1* mutations to these cancers (COSMIC database).

RAF1 and BRAF genes

The RAF1 (OMIM 164760), BRAF (OMIM 164757)and ARAF (OMIM 311010) proteins are members of a small family of serine-threonine kinases that function as RAS effectors.[7,172,173] These kinases phosphorylate and activate the dual specificity kinases MEK1 and MEK2, which in turn promote the activation of the MAPKs, ERK1 and ERK2. The three

members of the RAF family play different roles in the activation of the RAS-MAPK signaling cascade. BRAF has considerably higher MEK kinase activity compared to RAF1 and ARAF. These proteins also differ in their expression profiles as well as in the regulatory mechanisms controlling their function, and appear to have unique roles during development.[174–176] Consistent with these observations, somatic *BRAF* mutations frequently occur in malignant melanomas and in thyroid, colorectal and ovarian cancers (COSMIC database), while *ARAF* and *RAF1* missense changes are observed rarely in malignancies. These kinases are characterized by three functional domains, known as conserved regions 1 to 3 (CR1-3) (Figure 6). The *N*-terminal CR1 contains the region involved in GTP-RAS binding and a cysteine-rich sequence that mediates protein interaction with the cytosolic surface of the cell membrane. CR2 contains a negative regulatory domain controlling protein translocation to the membrane and its catalytic activation, while the *C*-terminal CR3 comprises the kinase domain of the protein.[172]

We and another group identified missense mutations in *RAF1* (OMIM 164760) in subjects with NS with a prevalence of mutations ranging between 5–15% (Figure 2).[29,177] Mutations in the same gene were also identified in two of six subjects with LS without a mutation in *PTPN11*.[29] *RAF1* mutations affect residues clustering in three regions of the protein (Figure 6). The first cluster (70% of total *RAF1* defects) involves the *N*-terminal consensus 14-3-3 recognition sequence or adjacent residues within the CR2 region. Of note, Arg256, Ser257, Ser259 and Pro261, which are the invariant residues within this motif, were all found to be mutated. The second group (15% of *RAF1* lesions) includes mutations affecting residues within the activation segment region of the kinase domain (Asp486 and Thr491). Of note, several *BRAF* missense mutations detected in solid tumors alter the activation segment, including some (Asp594Gly and Thr599Ile) homologous to those of *RAF1* identified in subjects with NS. Finally, the third cluster (15% of *RAF1* mutations) affect two adjacent residues (Ser612 and Leu613) located at the *C*-terminus. Phenotype analysis of the NS and LS subjects with *RAF1* mutations is notable for the observation that a large percentage of cases (75%) exhibit HCM, compared to HCM prevalence of 18% in the general NS population.[29,177] Moreover, this genotype-phenotype correlation seems to be allele-specific as HCM appears to be associated with mutations affecting the *N*-terminal 14-3-3 consensus site or the *C*-terminus. In addition to the two cases with LS, multiple nevi, lentigines and/or café-au-lait spots were detectable in one-third of NS patients with *RAF1* mutations, suggesting a predisposition to hyperpigmented cutaneous lesions.

In RAF1's inactive state, the *N*-terminal portion of the protein interacts with and inactivates the kinase domain at the *C*-terminus. This autoinhibited conformation is stabilized by 14-3-3 protein dimers that bind to phosphorylated Ser259 and Ser621.[7,172,173] Dephosphorylation of Ser259, which is possibly mediated by protein phosphatase 1C (PP1C) or protein phosphatase 2A (PP2A), is required for stable interaction with GTP-RAS, allowing protein translocation to the plasma membrane and phosphorylation of regulatory residues required for RAF1 catalytic activation. Functional characterization of a selected panel of RAF1 mutants supports the idea that mutations can differentially perturb protein function and intracellular signaling. In particular, mutations affecting the *N*-terminal 14-3-3 binding motif or the *C*-terminus of the protein, promote enhanced kinase activity and increased activation of the MAPK cascade compared to wild-type protein.[29,177] In contrast, Asp486Asn and Thr491Ile, representing the mutation cluster in the activation segment, were observed to be kinase impaired and differentially perturb signaling through the MAPK cascade. Pandit and co-workers provided evidence supporting the model that the increased activation promoted by the amino acid substitutions affecting the *N*-terminal 14-3-3 binding site results from a loss of 14-3-3-mediated inactivation,[29] while the mechanism through which mutations at the *C*-terminus activate RAF1's kinase activity remains to be explained.

Two groups independently discovered that *BRAF* is a major disease gene for CFCS (50–75% of cases) (Figure 2).[56,57] More recently, germline *BRAF* mutations have been documented also in a small percentage of subjects with phenotypes fitting or suggestive of NS or LS (< 2% of cases) (Figure 2).[30,31,177,178] Of note, NS-associated mutations largely do not overlap with those occurring in CFCS, suggesting a genotype-phenotype correlation. The recurrence of the Trp531Cys amino acid substitution as well as the clustering of mutations at the Thr^{241} further supports the idea that the phenotype resulting from germline *BRAF* defects might be allele specific. Among subjects with NS, the available clinical data indicate an association with neonatal growth failure and feeding difficulties, mild-to-moderate cognitive deficits, and hypotonia, and a higher prevalence of multiple nevi and dark colored lentigines.[30] As adults, they display the full-blown phenotype of NS, which is more severe compared to those associated with *PTPN11* and *SOS1* mutations. In these individuals, however, polyhydramnios, HCM, and CFCS-related skin features are uncommon or absent,[52,179] and cardiac defects, neurological impairment and feeding problems appear to be less severe compared to what is generally observed in CFCS.[30] While the occurrence of distinct *BRAF* gene mutations in subjects with a phenotype fitting NS would indicate that some features might be allele-specific, the observation that a subgroup of *BRAF* mutation-positive subjects exhibits an “intermediate” phenotype suggests that a clinical continuum characterized by a differential combination and severity of features is associated with defects in the *BRAF* gene.[30] These findings emphasize the difficulty in identifying efficient clinical criteria to define CFCS, LS or NS nosologically, and make evident the usefulness of a molecular-based definition of these clinically overlapping conditions that might direct clinicians towards the appropriate management of patients. Of note, the observation of a patient with LS and normal intelligence who was found to carry a novel sequence change in *BRAF*,[31] further illustrates that the phenotypic spectrum caused by *BRAF* mutations is broader than previously assumed and that mental retardation is not an invariably associated feature.

More than 40 germline *BRAF* mutations have been identified (Figure 6). All are missense defects that are not randomly distributed, clustering in the cysteine-rich domain and in the amino-terminal portion and activation segment of the kinase domain. Most mutations are recurrent, with substitutions of residues Gln257 and Glu501 accounting for approximately 40% of all defects. Germline *BRAF* mutations are rarely observed as somatic mutations contributing to oncogenesis and NIH-3T3 cell colony focus formation assay data indicate that CFCS-associated BRAF mutants have a reduced transforming capability compared with the recurrent oncogenic Val600Glu BRAF protein, and that the NS- and LS-associated BRAF mutants do not confer enhanced transformation to cells.[30] While these results support the view that the mutations transmitted through the germline have less potency in deregulating BRAF’s function, they also suggest that NS- and LS-associated mutations are less activating compared to those associated with CFCS, which parallels observations indicating that phenotypes arising from germline *BRAF* defects might be allele specific. Of note, *in vitro* functional studies indicate that germline and somatic amino acid changes can confer either increased or decreased activity upon the mutant protein,[30,57,180] suggesting that multiple alternative mechanisms are likely to be involved in the functional dysregulation of the kinase.

SHOC2 gene

We recently discovered that the invariant 4A>G missense (Ser2Gly) change in the *SHOC2* gene (OMIM 602775), which encodes a cytoplasmic leucine-rich repeat-containing protein that positively modulates RAS-MAPK signal flow,[181–185] underlies NS/LAH.[39] This amino acid change was demonstrated to promote *N*-myristoylation of the protein, and causes aberrant targeting of SHOC2 to the plasma membrane and impaired translocation to the

nucleus upon growth factor stimulation (Figure 7).[39] *N*-myristoylation is an irreversible form of protein fatty acylation occurring during protein synthesis, in which myristate, a 14-carbon saturated fatty acid, is covalently added to an *N*-terminal glycine residue after excision of the initiator methionine residue by methionylaminopeptidase.[186–188] It is a relatively common lipid modification of many signal transducers, and contributes to direct protein anchoring to cellular membranes, which is required for their proper function.

SHOC2 is a widely expressed protein composed almost entirely by leucine-rich repeats (LRR) and has a lysine-rich sequence at the *N*-terminus (Figure 7). Since LRRs can provide a structural framework for protein-protein interactions, SHOC2 is believed to function as a scaffold linking RAS to downstream signal transducers. It has recently been reported that SHOC2 functions as a regulatory subunit of the catalytic subunit of protein phosphatase 1 (PP1C).[184] By binding GTP-MRAS, SHOC2 promotes PP1C translocation to the membrane, allowing PP1C-mediated RAF1 dephosphorylation at residue Ser259, which is required for stable RAF1 translocation to the plasma membrane and catalytic activation. According to this model, constitutive membrane translocation of the disease-causing $SHOC2^{S2G}$ protein is expected to promote prolonged PP1C-mediated RAF1 dephosphorylation at Ser259 and, consequently, sustained RAF1-stimulated MAPK activation. Initial functional characterization of the mutant protein indicates that the expression of $SHOC2^{S2G}$ mutant enhances ERK activation in a cell type-specific fashion.[39] The observations that subcellular localization of SHOC2 is restricted to the nucleus following EGF stimulation and that $SHOC2^{S2G}$ ectopic expression in *C. elegans* engenders protruding vulva, a neomorphic phenotype previously associated with aberrant signaling, suggest that the disease-causing mutant might exert a wider perturbing effect on intracellular signaling.

MEK1 and MEK2 genes

MEK1 (OMIM 176872) and the functionally related MEK2 (OMIM 601263), belong to a family of dual specificity kinases that phosphorylate ERK proteins at tyrosine and serine/threonine residues.[189] The MEK proteins share a conserved structure including a negative regulatory domain at the *N*-terminus and a single protein kinase domain. MEK1 and MEK2 are both effectors of RAF proteins, but appear to play non-redundant roles. In particular, genetic evidence from mouse models indicates that MEK1 function is required during embryonic development,[190] while MEK2 is dispensable.[191] Of note, even though MEK kinase activity is necessary for cell transformation via the MAPK cascade,[192] and constitutively active MEK mutants promote transformation,[193] mutations in these genes have not been reported in human cancers so far (COSMIC database). Missense mutations and in-frame deletions in *MEK1* and *MEK2* account for approximately 20% of CFCS.[57–60,178,194] Recently, one germline mutation in *MEK1*, predicting the Asp67Asn amino acid substitution, has been documented in two unrelated subjects exhibiting a phenotype fitting NS.[59] According to this study, *MEK1* gene mutations would account for approximately 3% of *PTPN11*- and *SOS1*-mutation negative NS cases. In NS and CFCS, *MEK* gene mutations are mostly missense changes affecting residues located in the regulatory region and the *N*-terminal portion of the catalytic domain. Functional characterization of a panel of CFCS-causing mutants documented that they are more active than the wild-type protein in stimulating ERK phosphorylation.[57,195] No datum on the effect of the Asp67Asn change on MEK1 function and MAPK signaling is currently available.

Concluding remarks

While dysregulation of the RAS-MAPK signaling pathway has been known for decades as a major event in oncogenesis, discoveries derived from a massive disease gene hunting effort have only recently established a novel scenario in which the upregulation of this signaling

cascade underlies a group of clinically related developmental disorders. Based on the relatively high prevalence of some of these disorders, the dysregulation of this signaling pathway also represents one of the most common events affecting developmental processes. These discoveries have also provided us with novel molecular mechanisms causing enhanced signaling.

During the last years, screening efforts and genotype-phenotype correlation analyses have documented that mutations in identified disease genes are associated with distinct phenotypes, explaining, in part, the wide clinical diversity characterizing these disorders. It should be considered that the overlapping features observed among disorders of the NCFCS family, the wide breadth of phenotypes within each trait, and absence of clinical features with pathognomic value and consensus on specific and routinely used diagnostic criteria make diagnosis of these diseases challenging, particularly during early infancy and in adulthood. Such a failure has direct impact on patient management because of the prognostic relevance of diagnosis (prevalence and extent of cardiac disease, degree of cognitive and growth impairment, cancer predisposition). Molecular diagnosis now offers the opportunity to overcome the weakness of subjective clinical criteria and represents a highly informative prognostic tool, with direct impact on counseling and appropriate patient management.

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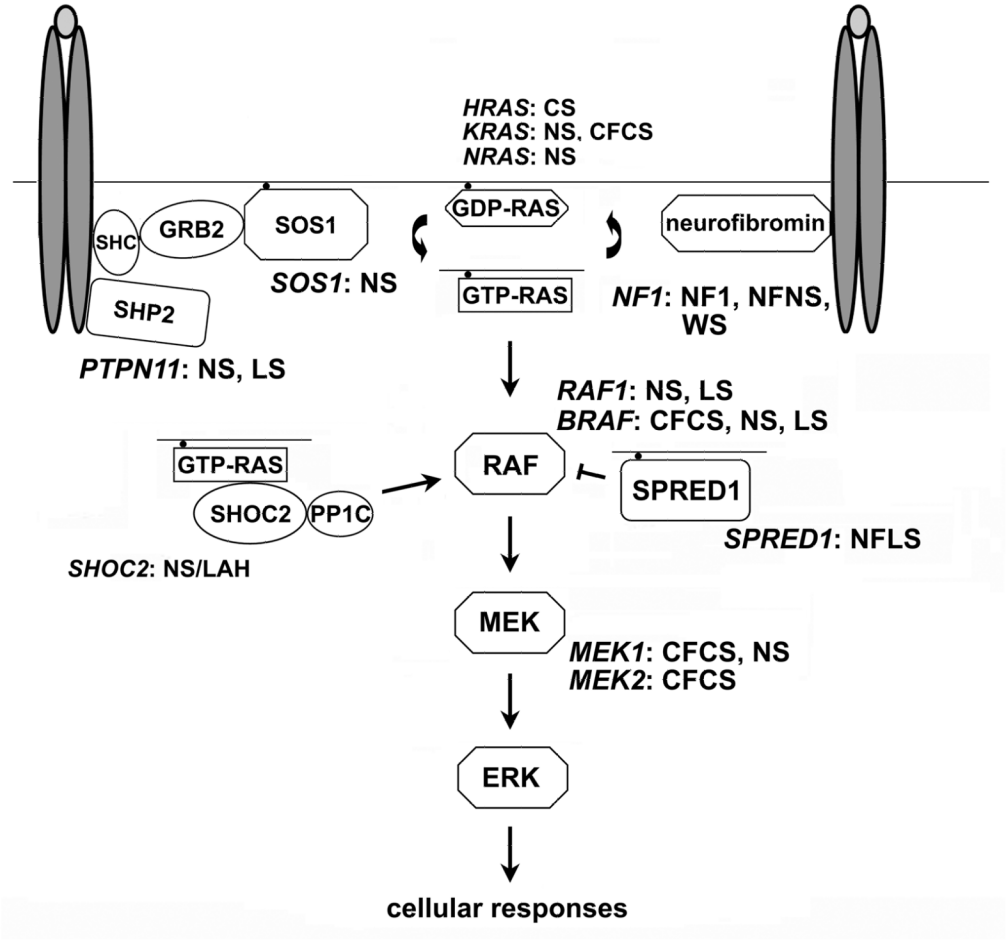

Figure 1. Schematic diagram showing the RAS-MAPK signal transduction pathway and affected disease genes in disorders of the neuro-cardio-facial-cutaneous syndrome family

The double ovals in dark grey and the light grey ovals represent generic dimerized cell-surface receptors binding to their ligand. Abbreviations: CFCS, cardio-facio-cutaneous syndrome; CS: Costello syndrome; LS, LEOPARD syndrome; NF1, neurofibromatosis type 1; NFLS, Neurofibromatosis type 1-like syndrome (also termed Legius syndrome); NFNS, Neurofibromatosis-Noonan syndrome; NS, Noonan syndrome; NS/LAH, Noonan-like syndrome with loose anagen hair; WS, Watson syndrome.

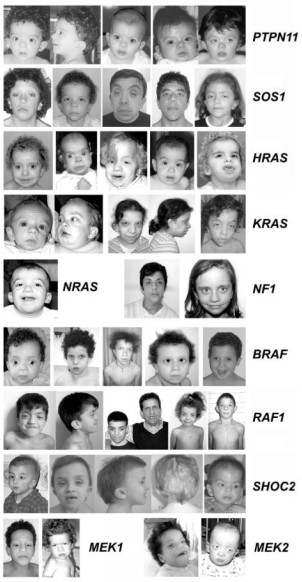

Figure 2. Dysmorphic facial features in disorders of the neuro-cardio-facial-cutaneous syndrome family caused by RAS-MAPK signaling dysregulation

Series of affected individuals heterozygous for mutations in different disease genes are shown [courtesy of G. Zampino (Università Cattolica del Sacro Cuore, Rome, Italy), B. Dallapiccola and M.C. Digilio (Ospedale “Bambino Gesù”, Rome, Italy), G. Mancini (Erasmus Medical Center, Rotterdam, The Netherlands), G.B. Ferrero (Università di Torino, Turin, Italy), and M. Zenker (University Hospital, Magdeburg, Germany)].

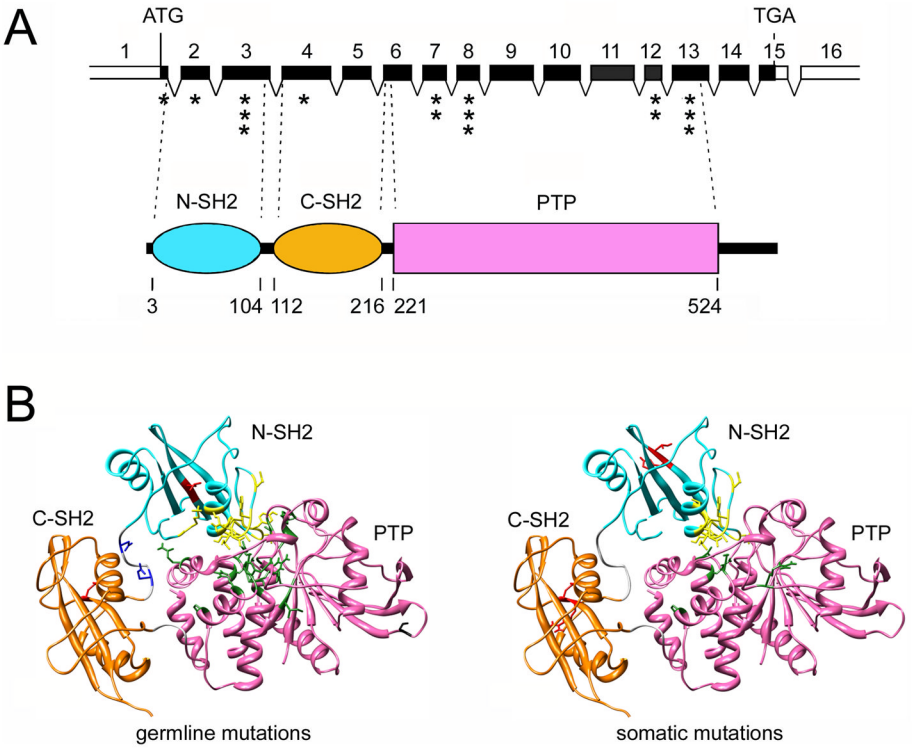

Figure 3. *PTPN11* gene organization, SHP2 domain structure and location of affected residues in human disease

(A) The *PTPN11* gene and its encoded protein product. The numbered, filled boxes at the top indicate the coding exons; the positions of the ATG and TGA codons are shown. Exon location of disease-associated mutations is indicated (the number of asterisks is an index of the relative prevalence of mutations within each exon). SHP2's functional domains, consisting of two tandemly arranged SH2 domains at the N-terminus (N-SH2 and C-SH2) followed by a protein tyrosine phosphatase (PTP) domain, are shown below. The numbers below that cartoon indicate the amino acid boundaries of those domains. (B) Location of mutated residues in the three dimensional structure of SHP2 in its catalytically inactive conformation.[94] Residues affected by germline (left) or somatically acquired (right) mutations are shown with their lateral chains (yellow, mutations affecting the N-SH2/PTP interaction; green, mutations affecting the N-SH2/PTP interaction and possibly substrate specificity and/or catalysis; red, mutations promoting increased SH2 phosphopeptide binding affinity or affecting specificity; blue, mutations affecting SH2 orientation or mobility; black, unclassified). The identity of affected residues and amino acid substitutions, and the prevalence of mutations are listed in Tartaglia *et al.*[98]

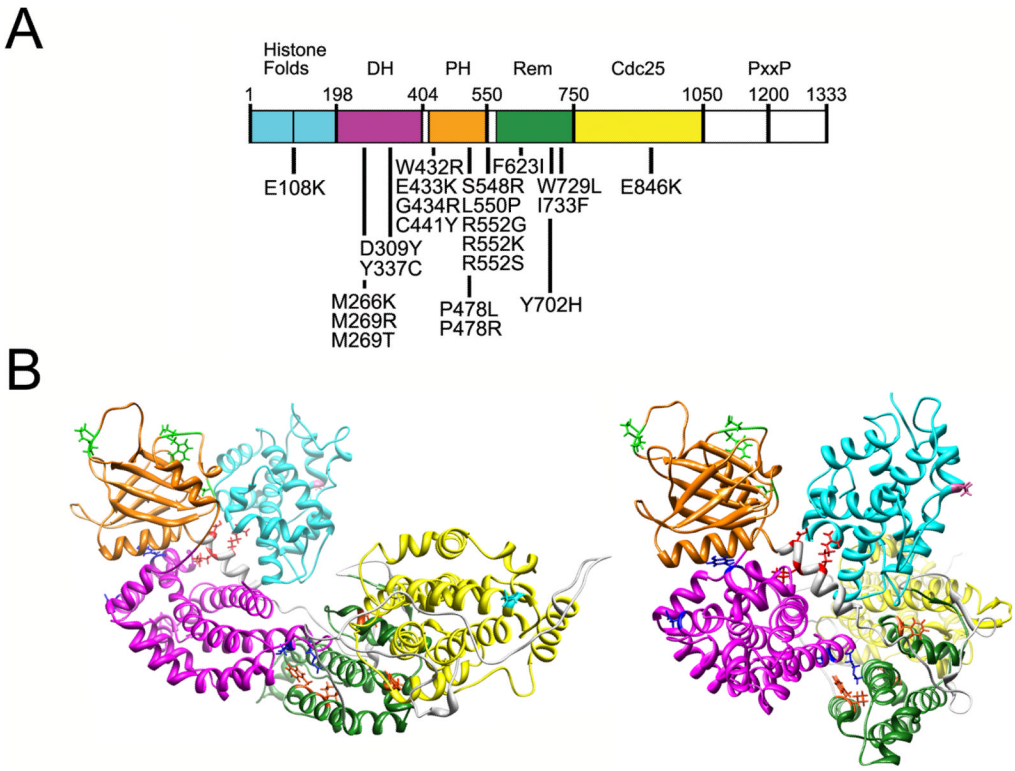

Figure 4. SOS1 domain structure and location of affected residues in NS

(A) *SOS1* missense mutations are positioned below the cartoon of the SOS1 protein with its functional domains indicated above. Abbreviations: DH, Dbl homology domain; PH, plekstrin homology domain; Rem, RAS exchanger motif; PxxP, proline-rich region. (B) Location of the mutated residues on the three-dimensional structure of SOS1. The functional domains are color coded as follows: Histone folds, cyan; DH, magenta; PH, orange; Rem, green; Cdc25, yellow. Residues affected by mutations are indicated with their lateral chains (histone folds, violet; DH, blue; PH, green; helical linker, red; Rem, orange; Cdc25, cyan). Based on Sondermann *et al.*[121], which utilized structural data and computational modeling.

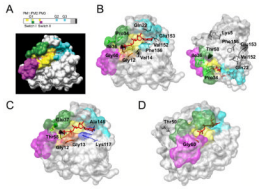

Figure 5. Domain structure of RAS proteins and location of affected residues in NS, CFCS and CS

(A) Schematic diagram and tridimensional representation of the structural and functional domains defined within RAS proteins. The motifs required for signaling function (PM1 to PM3 indicate residues involved in binding to the phosphate groups, while G1 to G3 are those involved in binding to the guanine base) are indicated. The hypervariable region is shown in grey, together with the *C*-terminal motifs that direct post-translational processing and plasma membrane anchoring (dark grey). The GTP/GDP binding pocket is shown in cyan (guanine ring binding surface) and yellow (triphosphate group binding surface) together with the Switch I (forest green) and Switch II (magenta) domains, according to the GTP-bound RAS conformation. (B) Location of the KRAS residues mutated in NS and CFCS. Residues affected by mutations are indicated with their lateral chains (triphosphate group binding surface, orange; guanine ring binding surface, blue; Switch I, green; other regions; black). (C) Location of the HRAS residues mutated in CS. Residues affected by mutations are indicated with their lateral chains as reported above. (C) Location of the NRAS residues mutated in NS. Residues affected by mutations are indicated with their lateral chains as reported above.

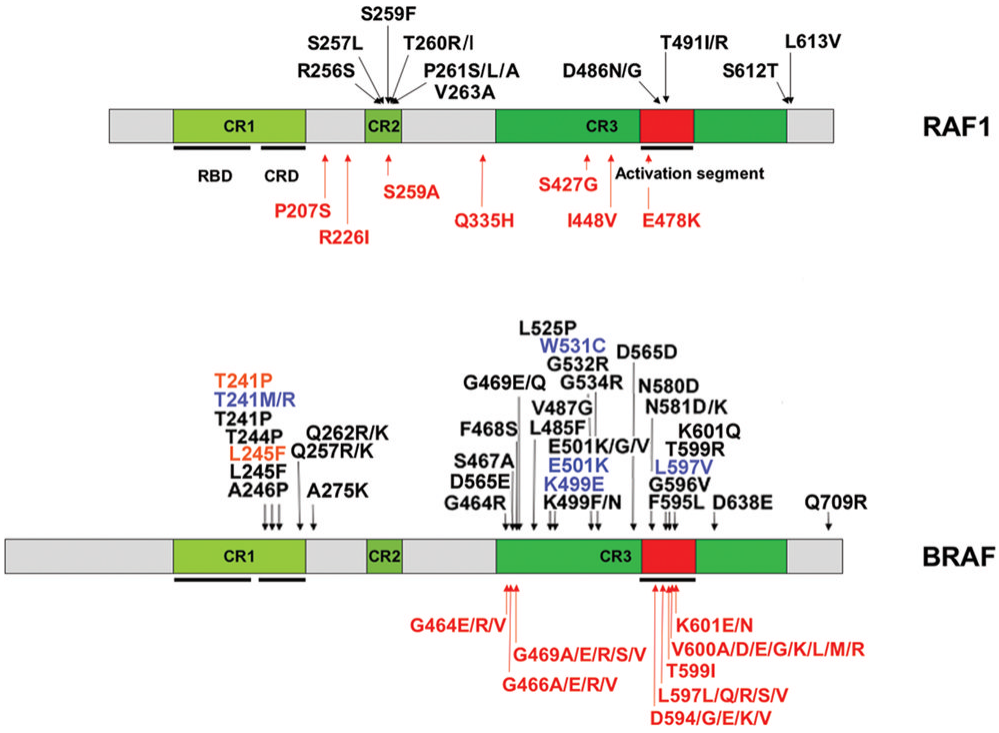

Figure 6. RAF1 and BRAF domain structure and location of affected residues in human disease

The domains of the RAF1 (above) and BRAF (below) proteins are indicated (CR, conserved region; RBD, RAS binding domain, CRD, cysteine-rich domain). Germline (RAF1: NS and LS; BRAF: NS, LS and CFCS) and somatic (associated with cancer) mutations are reported above and below each cartoon, respectively. Only somatic BRAF mutations with prevalence ≥ 1.5%, according the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/) are reported. The BRAF Thr599Ile substitution, which rarely occurs in cancer and is homologous to the NS-causing RAF1 Thr491Ile change, is also reported. BRAF missense changes associated with a phenotype fitting NS or LS are colored blue and orange, respectively.

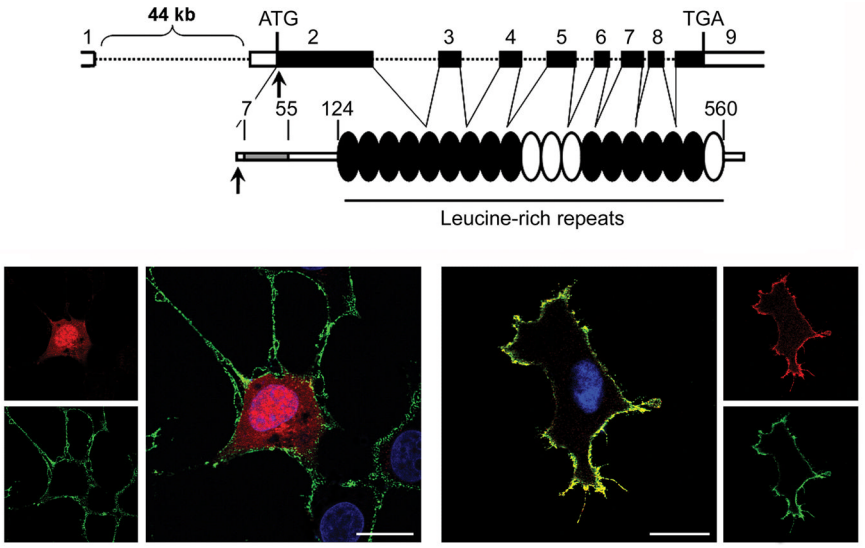

Figure 7. The disease-causing 4A > G (Ser2Gly) change in *SHOC2* promotes protein myristoylation and cell membrane targeting

(A) *SHOC2* genomic organization and protein structure. The coding exons are shown at the top as numbered filled boxes. Intronic regions are reported as dotted lines. SHOC2 motifs comprise an *N*-terminal lysine-rich region (grey coloured) followed by 19 leucine-rich repeats. Numbers above the domain structure indicate the amino acid boundaries of those domains. (B) Confocal laser scanning microscopy analysis documents that $SHOC2^{wt}$ (red) is uniformly distributed in the cytoplasm and nucleus (left) of transiently transfected Cos-1 cells (DMEM supplemented with 10% heat-inactivated FBS), while $SHOC2^{S2G}$ (red) co-localizes with ganglioside M1 (green) to the cell membrane.[39] Nuclei are visualized by DAPI staining (blue). Bars indicate 20μm.