The RASopathy Family: Consequences of Germline Activation of the RAS/MAPK Pathway

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ABSTRACT Noonan syndrome [NS; Mendelian Inheritance in Men (MIM) #163950] and related syndromes [Noonan syndrome with multiple lentigines (formerly called LEOPARD syndrome; MIM #151100), Noonan-like syndrome with loose anagen hair (MIM #607721), Costello syndrome (MIM #218040), cardio-facio-cutaneous syndrome (MIM #115150), type I neurofibromatosis (MIM #162200), and Legius syndrome (MIM #611431)] are a group of related genetic disorders associated with distinctive facial features, cardiopathies, growth and skeletal abnormalities, developmental delay/mental retardation, and tumor predisposition. NS was clinically described more than 50 years ago, and disease genes have been identified throughout the last 3 decades, providing a molecular basis to better understand their physiopathology and identify targets for therapeutic strategies. Most of these genes encode proteins belonging to or regulating the so-called RAS/MAPK signaling pathway, so these syndromes have been gathered under the name RASopathies. In this review, we provide a clinical overview of RASopathies and an update on their genetics. We then focus on the functional and pathophysiological effects of RASopathy-causing mutations and discuss therapeutic perspectives and future directions. ***(Endocrine Reviews 39: 676 – 700, 2018)***

Clinical Presentation of RASopathies

With a cumulative incidence of about 1 per 1000 live births, RASopathies represent one of the largest groups of developmental disorders. In addition to characteristic congenital features, various homeostatic defects in RASopathies have been documented in recent studies. In this section, we describe the phenotypic spectrum of RASopathies, taking Noonan syndrome (NS) as the prototype, then focus on specific traits that distinguish the other syndromes.

NS as a prototype

First described by the pediatric cardiologist Jacqueline Noonan 50 years ago, NS is among the most common genetic disorders, with an estimated prevalence between 1 per 2000 and 1 per 2500 live births (1). NS is an autosomal-dominant genetic disease characterized by a distinctive phenotypic triad: facial dysmorphic features, cardiopathies, and growth retardation, to which many other developmental defects can be added, including mild to moderate developmental delay/learning disabilities, skeletal abnormalities, cryptorchidism in males, predisposition to myeloproliferative disorders, and endocrine/metabolic imbalance. Both heterogeneity among patients with NS and redundancy with other RASopathies can make early diagnosis with certainty difficult (2, 3).

Patients with NS have *distinctive facial features* (*i.e.*, hypertelorism, ptosis, downslanting palpebral fissures, low-set posteriorly rotated ears, and short/webbed neck) that are most striking from the newborn period until middle childhood and become less prominent in adulthood (4).

Eighty percent of patients with NS display *congenital heart defects*, ranking it as the second–most common syndromic cause of congenital cardiopathies after trisomy 21. The most frequent heart defects are pulmonary valve stenosis (PVS; 60%) and hypertrophic cardiomyopathy (HCM; 20%), but many other anomalies, such as septal or valve defects, aortic coarctation and, persistently patent arterial duct, are found (5). Fatal heart failure/sudden death during the first year of life is a concern in up to one-fourth of patients with HCM (6). The high prevalence

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ESSENTIAL POINTS

- Noonan syndrome and related genetic diseases constitute one of the largest groups of developmental disorders and are associated, with variable penetrance and severity, with distinctive congenital defects (*i.e.,* facial features, cardiopathies, growth and skeletal abnormalities), developmental delay/mental retardation, and tumor predisposition
- In addition to congenital features, the recent identification of endocrine and metabolic dysfunctions pinpoints the need for an integrated view of the diseases rather than a symptom-directed exploration
- These diseases have been named RASopathies after the discovery of ~20 disease genes, almost all of which encode proteins belonging to or regulating the so-called RAS/MAPK signaling pathway
- Complementary functional analyses have demonstrated the role of RAS/MAPK hyperactivation in driving developmental abnormalities as well as endocrine and metabolic imbalances, although additional signaling dysregulations are also involved
- Despite genetic heterogeneity and complex phenotype/genotype correlations, the different syndromes seem to be characterized by the position of their causal genes within the RAS/MAPK pathway, with mutations hitting the backbone of the cascade associated with a more severe phenotype
- The identification of pathophysiological mechanisms provides insights into the development of specific therapeutic strategies, notably treatment aimed at reducing RAS/MAPK hyperactivation

and mortality of cardiac involvement justify complete cardiac exploration at diagnosis and during follow-up (7).

A cardinal feature of NS, which can lead to diagnosis, is *postnatal proportionate short stature*, reported in 80% of patients. Prenatal growth is classically normal, with birth weight and body length in the normal range. Growth failure usually occurs during early childhood and can result from feeding difficulties, justifying enteral nutrition in one-fifth of infants. During childhood, the mean height in both sexes follows the lower limit of the normal population [third percentile or −2 standard deviation score (SDS)] until the age of puberty, after which it declines further as a result of delayed puberty and attenuated pubertal growth spurts. The final adult height is around −2 SDS in both sexes (8–13). Some patients with NS display normal to elevated serum GH levels associated with low serum IGF-1 levels, suggesting postreceptor GH insensitivity, which has been proposed as an explanation for growth retardation (14).

In addition to growth retardation, low body mass index has been reported in patients with NS from childhood until adulthood. The prevalence of overweight and obesity is lower in patients with NS than in the general adult population (8, 12, 15, 16). This could be related to early feeding difficulties, possibly subsequent to impaired satiety control. However, one study reported changes in body composition affecting both adipose tissues and muscle mass despite normal caloric intake (16).

As mentioned previously, the onset of puberty is usually delayed in patients with NS, with a mean age at pubertal onset of 13.4 years for boys and 13.0 years for girls (17). Gonadal dysfunction with deficient spermatogenesis and impaired fertility has been reported in men with NS (18). This feature may be related to cryptorchidism, which is reported in up to 80% of males with NS, as well as to Sertoli cell dysfunction (19, 20) and could explain the sex ratio distortion in familial cases (21).

Skeletal abnormalities such as pectus and scoliosis are frequent findings in patients with NS, as well as decreased bone mineral density (22, 23).

Mild to moderate *developmental delay/learning disability* is identified in more than half of patients with NS, notably social and communication difficulties, attention deficit, and language impairment (24). However, mental retardation (IQ < 70) is uncommon in patients who have NS, and their intelligence is within the normal range (25, 26).

Easy bruising and bleeding tendency can be present, and it is recommended that baseline coagulation screening be carried out, especially before any major surgery. Both coagulation factors and platelet defects have been suggested to explain this bleeding diathesis (27, 28). Whether related diseases share these defects is unknown to date.

Along with an eightfold increased cancer risk, NS is associated with *cancer predisposition*, notably childhood cancers (29). In particular, a higher incidence of juvenile myelomonocytic leukemia (JMML), with frequent neonatal manifestations, has been described in NS (30, 31) (Table 1).

Related syndromes: variations from the prototype

Noonan syndrome with multiple lentigines (NS-ML), formerly named LEOPARD syndrome (according to the acronym, multiple *l*entigines, *E*CG conduction abnormalities, *o*cular hypertelorism, *p*ulmonic stenosis, *a*bnormal genitalia, *r*etardation of growth, and sensorineural *d*eafness), has an estimated prevalence of fewer than 1 per 100,000 live births. However, the phenotypic redundancy with other RASopathies can make the diagnosis more difficult (32). NS-ML is phenotypically very close to NS but has distinctive

Table 1. Clinical Features and Genetics of RASopathies

Disease	Epidemiology	Major Symptoms	Disease Gene
Noonan syndrome (NS) MIM #163950	1/2000	Dysmorphism	*PTPN11* (50%), *SOS1* (10%), *RAF1* (10%), *KRAS*, *NRAS*, *SHOC2*, *CBL*, *BRAF*, *SOS2*, *RIT*, *RRAS*, *RASA2*, *SPRY1*, *LZTR1*, *MAP3K8*, *MYST4*, *A2ML1*
	Autosomal dominant	Congenital cardiac defects (**PVS 60%,** HCM 20%)	Unknown: 20%–30%
	de novo (60%) or familial	Skeletal abnormalities, short stature, low BMI	
		Delayed puberty, hypogonadism, cryptorchidism	
		Developmental delay/learning disability	
		Bleeding defects (easy bruising)	
		Cancer risk, JMML	
Noonan syndrome with multiple lentigines (NS-ML, formerly LEOPARD syndrome) MIM #151100	1/100,000	Dysmorphism	*PTPN11* (90%), *RAF1* (5%), *BRAF*
	Autosomal dominant	**HCM 80%**	Unknown: 5%
		Moderate short stature (50%)	
		Skeletal abnormalities	
		Mild intellectual disability (30%)	
		Multiple lentigines	
		Deafness (20%)	
Noonan-like syndrome disorder with loose anagen hair (NS-LAH) MIM #607721	≈100 reported cases	Dysmorphism	*SHOC2*, *PPP1CB*
	Autosomal dominant	Cardiac defects (**mitral valve, septal defects)**	
		Short stature	
		Hyperactive behavior	
		Loose anagen hair	
Costello syndrome (CS) MIM #218040	1/400,000	**Coarse facial features**	*HRAS*
	Autosomal dominant	Congenital cardiac defects (HCM, arrhythmia)	
		Failure to thrive, short stature	
		Cutaneous defects (deep palmar and plantar creases)	
		Papillomas	
		Mental retardation	
		Malignant risk (15%)	
Cranio-facio-cutaneous syndrome (CFCS)	1/200,000	Dysmorphism	*BRAF* (60%), *MEK1* (10%), *MEK2* (10%), *KRAS*

(*Continued*)

Table 1. *Continued*

Disease	Epidemiology	Major Symptoms	Disease Gene
MIM #115150	Autosomal dominant	Congenital cardiac defects (PVS, HCM, arrhythmia)	Unknown: 15%
		Short stature	
		Ectodermal abnormalities	
		Mental retardation	
Type 1 neurofibromatosis (NF1), also named von Recklinghausen disease, MIM#162200	1/3000	Rarer congenital cardiopathies	*NF1* (>90%)
	Autosomal dominant	**Multiple café au lait spots and freckling**	
	Familial or *de novo*	**Neurofibromas**/benign tumors	
		Iris Lisch nodules	
		Mild learning disabilities	
Legius syndrome (LS), MIM#611431	≈200 reported cases	Multiple café au lait spots without **neurofibromas**	*SPRED1* (>90%)
	Autosomal dominant	Mild learning disabilities	

Major clinical traits for the different syndromes are listed (nonexhaustively), with distinctive symptoms in bold. For disease genes, mutation frequency is given for the most common mutations. The percentage of unknown genes corresponds to the number of patients with a firm diagnosis and negative genetic testing for all known disease genes.
Abbreviations: BMI, body mass index; LEOPARD, lentigines, ECG conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness; MIM, Mendelian Inheritance in Men.

features, in particular a high prevalence of hearing deficits (20%) and multiple pigmented skin lesions called lentigines, mostly starting at school age (90%) and explaining the disrespectful naming of the disease (32). In contrast with the higher prevalence of PVS in patients with NS, the vast majority of patients with NS-ML (80%) display HCM, which can develop during childhood (32–34). The high risk of heart failure or sudden death requires cardiologic follow-up (33, 35–37). Growth retardation seems to be milder than in NS, affecting only ~30% of patients with NS-ML (12, 32, 38, 39) (Table 1). Analyses of a small cohort of patients with NS-ML reported lower-than-average body mass index, with reduced adiposity for the few patients tested (40). Neurosensorial deafness seems to be more prominent in NS-ML than in NS.

Noonan-like syndrome with loose anagen hair (NS-LAH), also known as Mazzanti syndrome, is phenotypically close to NS, but patients display distinctive hyperactive behavior and pathognomonic hair anomalies. The incidence of specific cardiac defects, notably mitral valve and septal defects, is higher with NS-LAH than with NS (41).

Cardio-facio-cutaneous syndrome (CFCS) shares the major clinical features of NS (*e.g.*, heart defects, short stature, and facial features) but differs from NS mainly in the high frequency of ectodermal anomalies (*i.e.*, thin, curly, friable hair and hyperkeratotic skin changes) and constant mental retardation (42).

In patients who have *Costello syndrome (CS)*, facial features are coarser than in patients with other RASopathies. They also have skin abnormalities such as soft and loose skin with deep palmar and plantar creases and sparse curly hair. A distinctive and common feature associated with CS is the presence of benign cutaneous papillomas in the perinasal or/and perianal region. Congenital heart defects are frequent, most commonly HCM and arrhythmias. Similar to patients with CFCS, most patients with CS display severe mental retardation (42). Finally, CS has the highest cancer risk among RASopathies, with a cumulative incidence of tumors of 15%, including rhabdomyosarcoma and, less frequently, neuroblastoma and bladder carcinoma (29). This tumor predisposition justifies systematic tumor screening. Recently, increased energy expenditure (EE) has been described in patients with CS, which has been proposed as a cause of growth failure (43).

With an incidence of 1 per 3000 live births, *type I neurofibromatosis (NF1)* is the second–most frequent RASopathy, whereas *Legius syndrome (LS)* is a rare condition. These conditions show only partial overlap with NS, with rarer cardiac manifestations and facial features being found in only a subgroup of patients, referred to as neurofibromatosis–Noonan syndrome. These diseases share distinctive features, including multiple café-au-lait spots and freckling, bone malformations, and mild learning disabilities. In addition, NF1 is characterized by the presence of benign tumors (neurofibromas, optic pathway gliomas, and Lisch nodules) (44, 45).

Genetics of RASopathies

To date, RASopathies have been associated with mutations in ~20 genes. Most, if not all, genes encode proteins belonging to or regulating the so-called RAS/MAPK signaling pathway, providing a genetic foundation for their diagnosis and for explaining their pathophysiologies. However, genetic heterogeneity for a given syndrome on one side and allelism for several syndromes on the other side make classification harder. In this article, we describe the RAS/MAPK pathway and its pleiotropic roles during development and in homeostasis maintenance; we then summarize the different causal genes and mutations involved in RASopathies and their interconnections and provide some genotype/phenotype correlations.

The RAS/MAPK pathway

The RAS/MAPK cascade is a ubiquitous signaling pathway activated in response to a wide range of extracellular stimuli (*e.g.,* growth factors, hormones, cell/cell interaction) to modulate various cellular processes (*e.g.,* proliferation, survival, differentiation, migration, or metabolism), thereby adapting cell fate to modification of its environment (Fig. 1). Although this signaling pathway is often summarized as a well-established, straightforward cascade, reality goes far beyond this scholastic view, with many remaining gray areas and multiple levels of regulation, including transcriptional control, posttranslational modifications, protein/protein and protein/lipid interactions, subcellular compartmentalization, and crosstalk with other signaling pathways. In this section, we limit our comments to the components that are relevant to the RASopathies, while referring the readers to more comprehensive reviews for detailed information (46, 47).

A central node in this pathway is the small guanosine triphophatase (GTPase) RAS proteins, which switch from a guanosine diphosphate–bound inactive state to a guanosine triphosphate (GTP)–bound active state. RAS-GTP activates several effectors, most notably phosphatidylinositol 3-kinase (PI3K), phospholipase C, RAL guanine nucleotide exchange factor, novel Ras effector 1/Ras association domain family member 5, and the RAF kinases. RAS activation is a complex, tightly regulated mechanism for which a canonical, albeit nonexclusive, model can be given in the case of tyrosine kinase receptors (*e.g.,* epidermal growth factor or insulin receptors). Ligand binding to its cognate receptor triggers receptor dimerization and autophosphorylation on several tyrosine residues, creating binding sites for adaptor proteins such as GRB2 or GAB1. This allows recruitment of SOS, the RAS GTPase exchange factor, to the plasma membrane, which converts RAS to its active state. In addition to SOS, the tyrosine phosphatase SHP2 participates in RAS activation by dephosphorylating several inhibitory phosphotyrosines on docking proteins or on RAS itself (48–50). At the other side of the equation, GTPase-activating proteins (GAPs), such as NF1 and RASA1/p120RASGAP, catalyze GTPase activity of RAS to trigger its return to the inactive state, which can be further reinforced by additional inhibitory events, including receptor ubiquitination by the E3 ubiquitin ligase CBL or sequestration of activating proteins by SPROUTY proteins (51–55). However, such a view is only the tip of the iceberg of RAS regulation, which remains largely unknown given (1) the tremendous number of simultaneous or mutually exclusive interactions among RAS, its effectors, and its regulators; (2) the spatiotemporal and dynamic control of those interactions and of enzymatic activities; and (3) the specific context of the plasma membrane environment.

Among the various RAS-GTP effectors, RAF is the first kinase of the so-called MAPK cascade. Its full activation requires the dephosphorylation of an inhibitory residue by the PP1C phosphatase, which is activated by the SHOC2 protein. RAF phosphorylates and activates the MAPK ERK kinases (MEK) which in turn activate the MAPK Extracellular signal-Regulated Kinases (ERK). Ultimately, activated ERKs phosphorylate different cytoplasmic targets (*e.g.,* MAPK-interacting kinase, a translation regulator) and nuclear targets (*e.g.,* transcription factors, integrator, an RNA polymerase II–associated complex), which initiate appropriate cellular responses to the initial stimulus.

Roles of the RAS/MAPK pathway

Developmental roles

During organismal development, the RAS/MAPK pathway is one of the main pathways to transduce intracellular signals in response to all kinds of mitogens (*e.g.,* growth factors), thereby initiating proliferation, survival, and antiapoptotic programs. Its roles in development are notably highlighted by the profound effect of pharmacological inhibition or genetic invalidation of key actors of this pathway in multiple cell types and animal models. For instance, total invalidation of genes encoding KRAS, SHP2, BRAF, MEK1, or ERK2 result in embryonic lethality as a consequence of early defects such as abnormal placentation or embryonic layer patterning, fetal liver defects, or cardiac anomalies (56–61), and tissue-specific knockouts severely affect the development of the corresponding tissue/organ [for recent reviews, see (54, 62, 63)]. Importantly, dysregulation of RAS/MAPK–dependent developmental processes have profound pathophysiological consequences. Indeed, RAS/MAPK pathway hyperactivation following somatic mutations of genes encoding membrane receptors or actors of the RAS/MAPK pathway has been causally linked to a wide range of cancers, so that approaches

aimed at inhibition of RAS/MAPK activation are currently under evaluation for anticancer therapies (64). Furthermore, in the next section we show the multiple developmental consequences of germline RASopathy-associated mutations of the RAS/MAPK pathway.

Endocrine and metabolic functions

In addition to the essential roles of the RAS/MAPK pathway during development, evidence has accumulated for its critical functions in regulating organismal homeostasis, in particular the endocrine and metabolic systems. This homeostatic function of the RAS/MAPK cascade is twofold: On one side, it can mediate the response to many hormones acting through membrane RTK (receptors tyrosine kinase) (*e.g.,* insulin, leptin, GH), and on the other side, it can ensure the proper development of many endocrine tissues (*e.g.,* hypothalamus, adipose tissue, pancreas) through tight spatiotemporal regulation (65–67).

Regarding hormone responses, RAS/MAPK activation is required for the leptin-evoked activation of pro-opiomelanocortin neurons, thereby inhibiting food intake and increasing EE (68). Indeed, genetic or pharmacological inhibition of SHP2 or ERK2 in the hypothalamus is associated with leptin resistance in mice, resulting in hyperphagy, decreased EE, and obesity (68, 69). Conversely, hypothalamic expression of a hyperactive mutant of SHP2 results in resistance to obesity and improves glucose homeostasis, a phenotype linked to persistent ERK activation (70). Its roles in insulin signaling are more complex. Indeed, ERK1/2-mediated insulin receptor substrate phosphorylation blunts PI3K/AKT signaling in many insulin-sensitive tissues, thereby promoting insulin resistance (71). Consistent with this, *Erk1*$^{-/-}$ and liver-specific *Ptpn11*$^{-/-}$ mice both display increased insulin sensitivity (72–75); in addition, chronic treatment with the MEK inhibitor (PD0325901) improves insulin sensitivity in mice fed a high-fat diet and in ob/ob mice (76). In contrast, components of the RAS/MAPK pathway have been identified as modifiers of insulin sensitivity through regulation of specific transcriptional programs (77), and muscle invalidation of SHP2 results in insulin resistance, which is associated with RAS/MAPK hypoactivation (78). RAS/MAPK is also mobilized downstream from the GH receptor and negatively regulates the production of IGF-1, the mediator of GH in growth control, both *in vitro* and *in vivo* (79).

RAS/MAPK also has pleiotropic roles in the development and function of endocrine and metabolic tissues. For instance, RAS/MAPK signaling participates in the development of the nervous system, affecting the production of and/or the response to neuroendocrine signals (80). Regarding adipose tissue, studies in cellular models and knockout mice revealed an important role for ERK1 and SHP2 in adipogenesis, resulting in both poor lipid management and defective adipokine (*e.g.,* leptin) production (75, 81), whereas treatment with MEK inhibitors suggested a positive role for MAPK in lipolysis (82, 83). SHP2 and ERK1/2 also play a positive role in pancreatic β-cell development and insulin synthesis or secretion (67, 84). In addition, components of the RAS/MAPK pathway are involved in ovarian and testis development and/or function, as disruption of ERK1/2 in ovarian granulosa cells impairs female fertility (85), whereas invalidation of SHP2 in Sertoli cells alters spermatogenesis and reduces FSH and testosterone production (86).

Interestingly, at the crossroad between proliferation and homeostasis, studies in the context of cancer biology have also highlighted a logical role for RAS/MAPK in triggering metabolism rewiring to anabolic programs through various processes, including the Warburg effect, autophagy, and macropinocytosis (87–92).

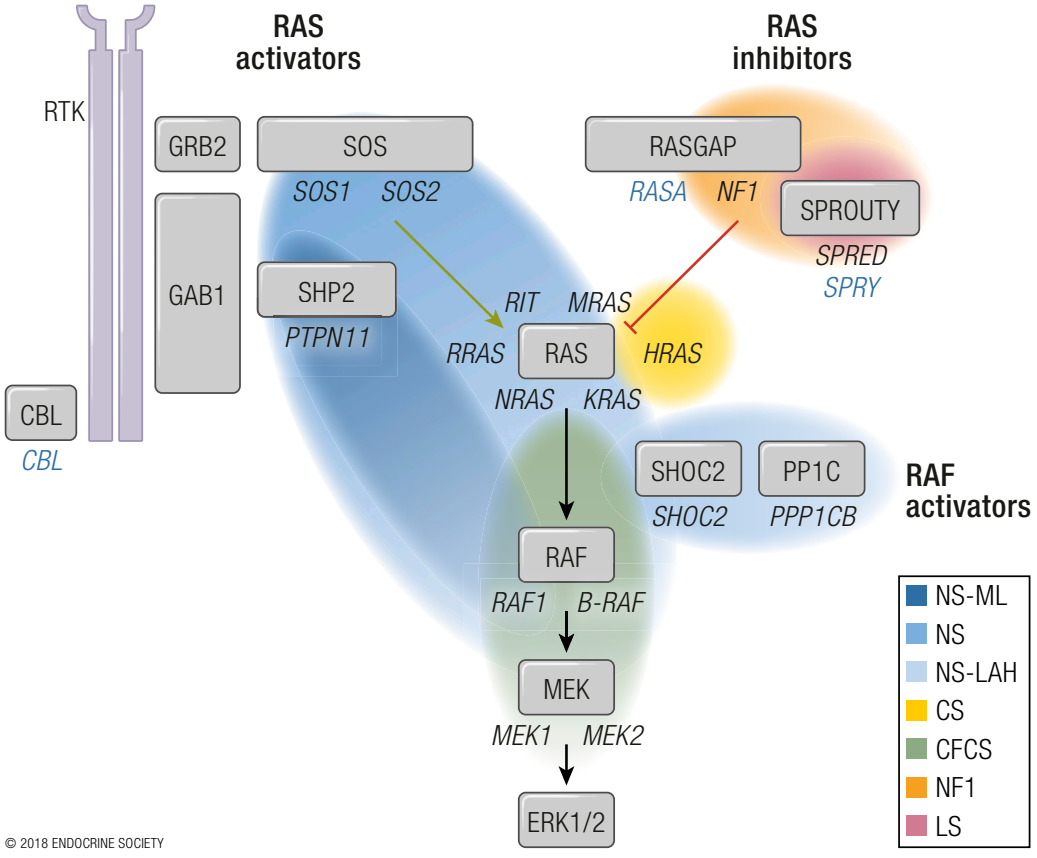
Germline mutations in the RAS/MAPK pathway, functional heterogeneity, allelism, and genotype/phenotype correlations

The first identified and major NS disease gene is the protein tyrosine phosphatase non-receptor type 11 (*PTPN11*), encoding SHP2, which is found mutated in 50% to 60% of patients with NS. Genetic screening efforts in patients with non-*PTPN11* NS revealed less common or rare mutations in a dozen genes related to the RAS/MAPK pathway [*SOS1* (10%), *RAF1* (10%), *KRAS*, *NRAS*, *BRAF*, *SHOC2*, *CBL*, *RIT*, *RRAS*, *RASA2*, *SOS2*, and *SPRY1*] to which several genes with yet unknown or RAS/MAPK-unrelated function can be added [*MAP3K8*, *MYST4*, *LZTR1*, and *A2ML1;* see (93) for a recent review]. Biallelic mutations in *LTZR1* were recently associated with an autosomal recessive form of NS (94). To date, 20% to 30% of patients with NS still lack a genetic diagnosis (93, 95). NS is thus the most genetically heterogeneous RASopathy, but also the most frequent, which may facilitate the identification of new disease genes and also allows the identification of genotype/phenotype correlations. Thus, when compared with *PTPN11*, *BRAF* mutations are associated with a more severe phenotype and a higher prevalence of HCM (96). Regarding cardiopathies, patients with *RAF1*, *RIT*, and *MRAS* mutations also develop more HCM (97–100), whereas *MEK1*, *KRAS*, and *SOS1* mutations tend to be highly associated with PVS (101). *SOS1/2* mutations are linked to a higher frequency of ectodermal anomalies and normal stature (12, 102, 103). The prevalence of cognitive/developmental delay is lower for *SOS1/2* mutations but higher for *RAF1*, *BRAF,* and *SHOC2* mutations (100). Finally, *RIT* and *CBL* mutations are associated with JMML, the latter also linked to low prevalence of cardiac defects, reduced growth, and cryptorchidism (104, 105).

The vast majority of patients with NS-ML (85%) harbor mutations within *PTPN11*. The remaining NS-ML cases are causally linked to *RAF1* and *BRAF*

"Patients with NS have distinctive facial features."

Figure 1. The RAS/MAPK pathway and the RASopathies disease genes. Simplified scheme depicting the canonical RAS/MAPK signaling pathway activated by receptors tyrosine kinase (RTK). Gray rectangles represent proteins (with corresponding genes italicized). Once activated by its ligand, an RTK autophosphorylates on tyrosine residues, creating binding sites for the adaptor protein GRB2 and the relocalization of SOS to the plasma membrane. RAS switches from a GDP-bound to a GTP-bound state, upon which it initiates the RAF>MEK>ERK phosphorylation cascade. RAS intrinsic GTPase activity hydrolyzes GTP to GDP, a reaction that is catalyzed by p120RASGAP. This signaling pathway is modulated at several levels: (1) SHP2 participates in RAS activation by dephosphorylating several inhibitory phosphotyrosines, such as the docking site for p120RASGAP borne by GAB1 and the tyrosine on RAS that mediates its interaction with p120RASGAP; (2) the scaffold protein SHOC2 mediates the activation of the PP1C phosphatase, which dephosphorylates an inhibitory residue on RAF; (3) SPROUTY proteins negatively regulate the RAS/MAPK pathway by sequestering RAS activators; and (4) CBL drives RTK ubiquitination, which turns off activating signals. Colored ovals encompass major disease genes for each RASopathy and reveal function/phenotype associations: In blue, NS and NS-like syndromes (NS-ML, NS-LAH) are caused mainly by mutations on RAS/RAF activators. In orange/pink, NF1 and LS are associated with RAS inhibitors. In yellow and green, mutations responsible for CS and CFCS affect the RAS/MAPK backbone. From a functional point of view, it seems that mutations hitting the low/central part of the signaling pathway give rise to more severe phenotypes. For better readability, some NS-associated genes are not covered by the blue oval but are written in blue, and disease genes unrelated to the RAS/MAPK pathway are not shown. CBL, Casitas B-lineage lymphomas; ERK, extracellular signal-regulated kinase; GAB1, Grb2 adaptor binder 1; GDP, guanosine diphosphate; GRB2, growth factor receptor-bound protein 2; GTP, guanosine triphosphate; MEK, MAPK/ERK kinase; PP, protein phosphatase; RAF, rapidly accelerated fibrosarcoma; SHOC2, Soc-2 homolog; SOS, son of sevenless.



mutations, which are associated with a more severe phenotype (96).

The main CFCS disease gene is *BRAF* (60%), but a substantial proportion of patients carry mutations on *MEK1* (10%), *MEK2* (10%), and *KRAS*. CFCS congenital heart defects, particularly mitral valve and septal defects, and ocular anomalies seem to be less frequent among patients with *MEK1/MEK2* mutations (106).

The other RASopathies are more genetically homogeneous, with mutations in the *HRAS* gene for almost all patients with CS, a unique mutation on *SHOC2*, and more recently mutations in *PPP1CB* found in patients with NS-LAH (41, 107), mutations in *SPRED1* for LS, and heterozygous loss-of-function (LOF) mutations or deletions of the *NF1* gene in more than 90% of NF1 cases (44, 45).

Adding further complexity to the genetic heterogeneity, several RASopathies are allelic diseases. Thus, *PTPN11* mutations are associated with NS (85%) and NS-ML (15%); *SOS1* mutations with NS and CS/CFCS; *KRAS* mutations with NS and CFCS; *BRAF* mutations with CFCS, CS, NS-ML, and NS; *RAF1* mutations with NS and NS-ML; and *MEK1* mutations with CFCS, CS,

and NS. This allelism identifies important signaling nodes and may explain the partial overlap and the phenotypic continuum between these related syndromes. However, we show in the next section that functional analyses also revealed syndrome-specific properties for several mutants, which certainly explains the uniqueness of each RASopathy.

Thus, from a functional point of view, specificities emerge for the different syndromes: NS, NS-ML, and NS-LAH are mainly associated with positive regulators of the RAS/MAPK cascade (*i.e.,* RAS or RAF activators), whereas NF1 and LS are linked to RAS inhibitors. In contrast, CS- and CFCS-causing mutations hit the backbone of the cascade, CS being centered on RAS and CFCS on downstream kinases (Fig. 1). At the risk of overstatement, a tendency emerges in which mutations hitting the central or low part of the cascade give rise to more severe conditions, which may reflect the fact that downstream effectors of the pathway are more unescapable, with higher effect of their dysregulation, than proximal actors, which receive several levels of regulation and can be bypassed.

Functional and Pathophysiological Consequences of RASopathy-Associated Mutations

Functional analyses have been performed for a growing number of mutations, both *in vitro* and in animal models of RASopathies, leading to the general concept of RAS/MAPK hyperactivation as a pathogenic mechanism. Thus, sustained RAS/MAPK activation has been causally linked to the abnormal development of several tissues, leading to some congenital symptoms (*e.g.,* cardiac or craniofacial defects), as well as altered response to different hormones, giving rise to endocrine dysfunctions (*e.g.,* growth hormone insensitivity and growth retardation). However, controversies exist, and the contribution of RAS/MAPK–independent dysfunctions has been demonstrated in several models, which may explain the differences between syndromes.

Functional consequences of RASopathy-causing mutations on the RAS/MAPK pathway

Complementary approaches in functional genetics have been and still are useful for delineating the consequences of RASopathy-associated mutations. Thus, *in silico* modeling and biochemical analyses led to the identification of structural changes or modifications of enzymatic activity, protein stability, or protein/protein interaction. Ectopic expression of mutated proteins in cellular models and analyses of patient cells, including skin fibroblasts and induced pluripotent stem cells (iPSCs), then allowed understanding of how the mutations alter signaling pathways and cellular functions. Finally, their global effect at the organismal level has been comprehended thanks to the development of animal models of the diseases, in invertebrates (*Caenorhabditis elegans*, *Drosophila*) as well as in vertebrates (*Xenopus*, zebrafish and mouse) [see (54, 95, 108, 109) for recent reviews and Table 2].

Approximately 70 different NS-causing *PTPN11* mutations that hit residues within or close to the SH2 and protein tyrosine phosphatase (PTP) domains and result in increased phosphatase activity through different mechanisms (*e.g.,* release of auto-inhibitory constraints, increased recruitability) have been identified [reviewed in (54)] (Fig. 2). Interestingly, some studies have started to identify mutation/phenotype correlations; for instance, mutations of Asp61 or Thr71 are preferentially associated with myeloproliferative disorders/JMML (30), whereas Leu261, Leu262, and Arg265 mutations are associated with milder form of NS (170). Coherent with the positive role of SHP2 on the RAS/MAPK pathway, functional analyses have shown that activating, NS-associated, SHP2 mutations hyperactivate ERK1/2. This effect has been repeatedly reported in different cell types *in vitro*, in the basal state as well as under stimulation by several agonists, and in different tissues/organs in animal models (114, 130, 171–174).

In contrast to NS, the pattern of NS-ML–associated *PTPN11* mutations is restricted to conserved residues within the PTP domain that are required for PTP activity (*e.g.,* Tyr279, Thr468, Gln510). All NS-ML–causing mutations collapse the phosphatase activity of SHP2 under both basal and stimulated states (151, 175–180). However, these mutations are not merely LOF because (1) they retain substantial activity and are stimulatable and (2) true LOF mutations of *PTPN11* are associated with a distinct syndrome named metachondromatosis (Mendelian Inheritance in Men #156250). The effect of NS-ML–associated *PTPN11* mutations on the RAS/MAPK pathway is highly debated. Indeed, consistent with the reduced phosphatase activity of the mutants, overexpression of NS-ML–causing SHP2 mutants in HEK cells or in zebrafish embryos has reduced ERK1/2 activation in response to several agonists (134, 177, 181). Moreover, a mouse model of NS-ML carrying the Y279C mutation on SHP2 (*Ptpn11*$^{Y279C/+}$) also displayed hypoactivation of ERK1/2 in the heart in response to insulin or IGF-1 (151). In contrast, different reports highlighted that NS-ML–associated SHP2 mutants can have a dominant positive effect on RAS/MAPK activation. Indeed, expression of NS-ML mutants in flies and zebrafish hyperactivated ERK1/2 to a similar extent as NS-associated mutants did, interestingly enough resulting in a comparable phenotype for both types of mutations (114, 115, 130). Moreover, basal or agonist-induced MEK/ERK1/2 hyperactivation was measured in cells overexpressing NS-ML mutants in an original model of iPSC-derived

Table 2. Complementarity of Animal Models of RASopathies

Disease	Mutation	Strategy	Expression Pattern	Phenotype	Mechanisms	Reference
Caenorhabditis elegans						
NS-ML	*Ptp-2 LOH*	Deletion	Vulva	Semisterility/vulval induction		(110)
NS-LAH	*Shoc*^{S2G}	DNA microinjection	Vulva	Protruding vulva	Increased RAS/MAPK	(107)
CS	*Let-60/Ras GOF*	DNA microinjection	Vulva	Multivulva	Increased RAS/MAPK	(111)
CFCS	*Mek2 GOF*	DNA microinjection	Vulva	Multivulva	Increased RAS/MAPK	(112)
Drosophila						
NS	*Csw*$^{D61Y,\ E76K}$	Overexpression	Mushroom body neurons	Long-term memory defects	Increased RAS/MAPK	(113)
	Csw$^{A72S,\ N308D,\ E76K}$	Overexpression	Wing, eye	Ectopic veins	Increased RAS/MAPK	(114)
NS-ML	*Csw*$^{Y279C,\ T468M}$	Overexpression	Wing, eye	Ectopic veins	Increased RAS/MAPK	(115)
NS/CS/ CFCS	*Ras*^{R68Q}	Mutagenesis	Eye, wing, glia	Ectopic veins, resistance to cell death	Increased RAS/MAPK	(116)
CS	*Ras*^{G12V}	Overexpression	Heart	Cardiac hypertrophy	Increased RAS/MAPK	(117)
NF1	*Nf*$^{-/-}$	Insertion of P element	Ubiquitous	Reduced pupae size, shortened life span, abnormal circadian rhythm	Increased RAS/MAPK, mitochondria ROS, impaired cAMP	(118–120)
			Nervous system	Defective in memory acquisition	Impaired cAMP	(121–123)
			Postmitotic larval brain neurons	Reduced growth, synaptic overgrowth	Impaired cAMP	(124, 125)
Xenopus						
NS	*Ptpn11*^{N308D}	mRNA injection	Heart	Rescue of ΔShp2-dependent cardiac defects		(126)
CS	*CA Ras*	mRNA injection	Ubiquitous	Mesoderm induction during early embryogenesis	Increased RAS/MAPK	(127)
CFCS	*CA Raf or Mek*	mRNA injection	Ubiquitous	Mesoderm induction during early embryogenesis	Increased RAS/MAPK	(127–129)
Zebrafish						
NS	*Ptpn11*$^{D61G,\ T73I}$	mRNA injection	Ubiquitous	Craniofacial and heart defects	Increased RAS/MAPK Src dependent signaling	(130, 131)
	NRas^{I24N}	Synthetic RNA injection	Ubiquitous	Gastrulation and craniofacial defects	Increased RAS/MAPK	(132)
	KRas^{N116S}	Synthetic RNA injection	Ubiquitous	Craniofacial dysmorphia, heart defects	Increased RAS/MAPK	(133)
NS-ML	*Ptpn11*$^{A462T,\ G465A,\ Y279C,\ T468M}$	mRNA injection	Ubiquitous	Craniofacial and heart defects	Increased RAS/MAPK Src dependent signaling, PZR dependent	(130, 131, 134–136)
CS	*Ras*^{G12V}	mRNA injection	Ubiquitous	Tumors, reduced size and life span, smaller heart, and craniofacial defects in adult fish		(137)

(*Continued*)

Table 2. *Continued*

Disease	Mutation	Strategy	Expression Pattern	Phenotype	Mechanisms	Reference
CFCS	*BRaf, Mek1/2 mutations*	Microinjection of patient-derived mRNA	Ubiquitous	Developmental abnormalities	Increased RAS/MAPK	(138)
NF1	*Nf1a/b knockdown*	Morpholinos	Heart/vessels	Cardiovascular defects	Increased RAS/MAPK	(139)
	Nf1a/b$^{-/-}$	Targeted mutagenesis	Neurons	Myelination defects, learning deficits	Increased RAS/MAPK	(140, 141)
Mouse						
NS	*Ptpn11*^{D61G}	KI	Germline	Short stature, craniofacial defects, cardiac defect, MPD, cognitive deficits	Increased RAS/MAPK	(79, 142)
	Ptpn11^{N308D}	KI	Germline	Cardiac defects, growth retardation, craniofacial defects, hematological problems in homozygous	Increased RAS/MAPK	(143)
	Ptpn11^{D61Y}	Conditional KI	Germline	Severe cardiac defects	Increased RAS/MAPK	(143)
			Neural crest	Craniofacial defects	Increased RAS/MAPK	(143)
			Endothelial	Cardiac defects	Increased RAS/MAPK	(143)
			Myocardial	No phenotype	Increased RAS/MAPK	(143)
			Forebrain	Cognitive deficits	Increased RAS/MAPK	(144)
	Ptpn11^{D61A}	Conditional KI	Neuronal	Obesity resistance (in female)	Increased p38	(70)
	Ptpn11^{Q79R}	Additive transgenesis	Myocardial adult	No phenotype	Increased RAS/MAPK	(145)
			Myocardial fetal	Cardiac defects	Increased RAS/MAPK	(145)
		Conditional transgenic	Neural crest	Craniofacial defects	Increased RAS/MAPK	(146)
			Endothelial	Cardiac defects	Increased RAS/MAPK	(146)
	Sos1^{E846K}	Conditional KI	Germline	Cardiac defects, growth retardation, craniofacial defects, hematological problems in homozygous	Increased RAS/MAPK, Rac, and Stat3	(147)
	Raf^{L613V}	Conditional KI	Germline	Cardiac defects (HCM), growth retardation, craniofacial defects, hematological problems	Increased RAS/MAPK	(148)
			Endothelial	HCM	Increased RAS/MAPK	(149)
			Cardiomyocyte	Impaired contractility	Increased RAS/MAPK	(149)
	K-Ras^{V14I}	Conditional KI	Germline	Cardiac defects, growth retardation, craniofacial defects, hematological problems	Increased RAS/MAPK	(150)
NS-ML	*Ptpn11*^{Y279C}	Conditional KI	Germline	HCM, growth delay, skeletal defects, dysmorphia, abnormal genitalia, sensorineural defects	Increased AKT/mTOR	(151)
			Endothelial	Trabeculation and valvular hyperplasia	Increased AKT/mTOR	(152)
			Cardiomyocyte	Ventricular septal defects	Increased AKT/mTOR	(152)

(*Continued*)

Table 2. *Continued*

Disease	Mutation	Strategy	Expression Pattern	Phenotype	Mechanisms	Reference
	Ptpn11^{T468M}	KI	Germline	HCM, growth delay, dysmorphia, reduced adipogenesis, increased EE, improved insulin sensitivity	Increased RAS/MAPK and AKT/mTOR	(40)
	Ptpn11^{Q510E}	Additive transgenesis	Myocardial fetal	HCM	Increased mTOR	(153)
			Myocardial adult	No phenotype	Increased mTOR	(153)
			Endothelial	Increased atrioventricular endocardial cushions	Increased mTOR	(154)
CS	*H-Ras*^{G12V}	Conditional KI	Germline	Model 1: facial dysmorphia, HCM, systemic hypertension, vascular remodeling, fibrosis, neurocognitive deficits, no cancer	No change in RAS/MAPK and Akt	(155)
				Model 2: cranial dysmorphia, no heart defects, tumors		(156)
CFCS	*B-Raf*^{V600E}	Conditional KI	Germline	Reduced life span, growth defect, facial dysmorphism, cardiomegaly, epileptic seizures, neuroendocrine tumors	No change in RAS/MAPK	(157)
	B-Raf^{L597V}	Conditional KI	Germline	Short stature, facial dysmorphia, HCM	Increased RAS/MAPK	(158)
	B-Raf^{Q241R}	Conditional KI	Germline	Embryonic/neonatal lethality, heart defects, liver necrosis, edema, craniofacial abnormalities, lymphatic defects	Increased RAS/MAPK, decreased p38 and AKT	(159)
	Mek1^{Y130C}	KI	Germline	Pulmonary artery stenosis, cranial dysmorphia, neurologic anomalies	Increased RAS/MAPK	(160)
NF1	*Nf*$^{+/-}$	KO	Germline	Mild bone defects, deficits in learning, pheochromocytomas (embryonic lethality for *Nf*$^{-/-}$)	PI3K/mTOR/AKT pathway	(161, 162)
	Nf $^{-/-}$	Conditional KO	Endothelial	Cardiac defects	PI3K/mTOR/AKT pathway	(163)
			Myocardial	Cardiac defects	RAS/MAPK pathway	(164)
			MSC	Skeletal malformations	RAS/MAPK pathway	(165)
			Neuronal	Brain malformation, learning deficits	PI3K/mTOR/AKT pathway	(166)
			Schwann cells	Neurofibromas	PI3K/mTOR/AKT pathway	(167)
			Myeloid	JMML	PI3K/mTOR/AKT pathway	(168)
LS	*Spred1*$^{-/-}$	KO	Germline	Facial dysmorphia, deficits in learning and memory, smaller body weight, hematologic alteration	Increased RAS/MAPK and JAK2	(169)

Because of their simple morphology and short life cycle, *C. elegans* and *Drosophila* are useful for high-throughput and quantitative studies; *Xenopus* and zebrafish help monitor developmental defects. Mice are commonly used to reproduce phenotypes of human RASopathies.
Abbreviations: CA, constitutively active; GOF, gain of function; KI, knockin; KO, knockout; MPD, myeloproliferative disorder.

cardiomyocytes from patients with NS-ML and in tissues from mice expressing NS-ML–causing SHP2 mutants (*Ptpn11*^{Q510E}, *Ptpn11*$^{T468M/+}$) (40, 153, 180, 182). Although unexpected from biochemical studies, this gain-of-function effect of NS-ML mutants on RAS/MAPK activation could result from their increased sensitivity to activating stimuli and sustained binding to upstream regulators, which could counterbalance their residual activity, thereby conferring signal-enhancing properties on them (180). The fact that the NS-ML–associated SHP2 mutant can hyperactivate RAS/MAPK, at least upon some conditions that remain to be identified, could explain the phenotypic similarities between NS-ML and NS and other RASopathies.

Various mutation clusters destabilizing regions of the SOS proteins that contribute structurally to the maintenance of their autoinhibited state were found in *SOS1* and to a lesser extent in *SOS2* as causes of NS (102, 103). One mutation (T158A) was also identified in patients with CS/CFCS (183). Functional studies revealed a higher level of GTP-bound RAS and higher ERK1/2 phosphorylation in cells expressing SOS1 and SOS2 mutants (103, 184–187), as well as in cardiac tissues from mice expressing the NS-associated E846K variant of SOS1 (147). Whether NS-causing and CS/CFCS–causing SOS mutants differentially affect SOS activity or affect additional mechanisms is unknown; however, it was recently shown that NS-causing *SOS1* mutations hitting distinct functional domains differentially modified SOS affinity for plasma membranes (188).

Mutations of the different members of the RAS GTPase family have been found in the nucleotide binding site or in structural domains involved in its inactive-to-active conformational change. They give rise to reduced GTPase activity or favor the active conformation, respectively. Thus, CS-associated *HRAS* mutations affect mainly Gly12 or Gly13, which reduced its GTPase activity, resulting in active RAS accumulation and ERK1/2 hyperactivation. Sustained RAS/MAPK activation was also detected in tissues from CS mice (*HRas*$^{G12V/+}$), notably in the liver and the brain (155, 156, 189). Interestingly, another mutation in *HRAS*, resulting in aberrant *HRAS* transcript processing and modification of its subcellular localization, was recently identified and is associated with a distinctive, mild CS phenotype (190). *KRAS* mutations, associated with NS, CS, and CFCS, also lead to RAS/MAPK hyperactivation *in vitro* through different molecular mechanisms, ranging from reduction of its GTPase activity to GAP hyposensitivity or GTPase exchange factor hypersensitivity. Functional studies also suggest that CS-causing and CFCS-causing *KRAS* mutations are stronger than NS-associated modifications (191, 192). *KRas*$^{V14I/+}$ NS mice also display RAS/MAPK hyperactivation in some tissues (150). Mutations of *RIT*, *RRAS*, *NRAS*, and *MRAS*, more recently found in a small fraction of patients with NS, all result in decreased GTPase activity and subsequent RAS/MAPK hyperactivation (97, 193–196).

For the RAF proteins, mutations affect distinct domains for *RAF1* and *BRAF*. For both proteins, mutations can occur in the kinase domain, also called conserved region (CR) 3. Specific mutations in the CR1 domain of BRAF, which are involved in the RAS/BRAF interaction, have also been identified, and several *RAF1* mutations cluster in the CR2. The latter are located close to a regulatory phosphorylation site involved in an inhibitory interaction with the 14-3-3 protein. Surprisingly, although most of the mutations are activating and result in RAS/MAPK hyperactivation *in vitro* and *in vivo*, several BRAF and RAF1 mutants are kinase impaired. Wu *et al.* (197) demonstrated that such defective RAF1 mutants display increased capability to heterodimerize with BRAF, resulting in a net increase of the dimer. Comparative analysis suggests that CFCS-associated *BRAF* mutations are stronger than those causing NS or NS-ML (138, 157–159, 198, 199).

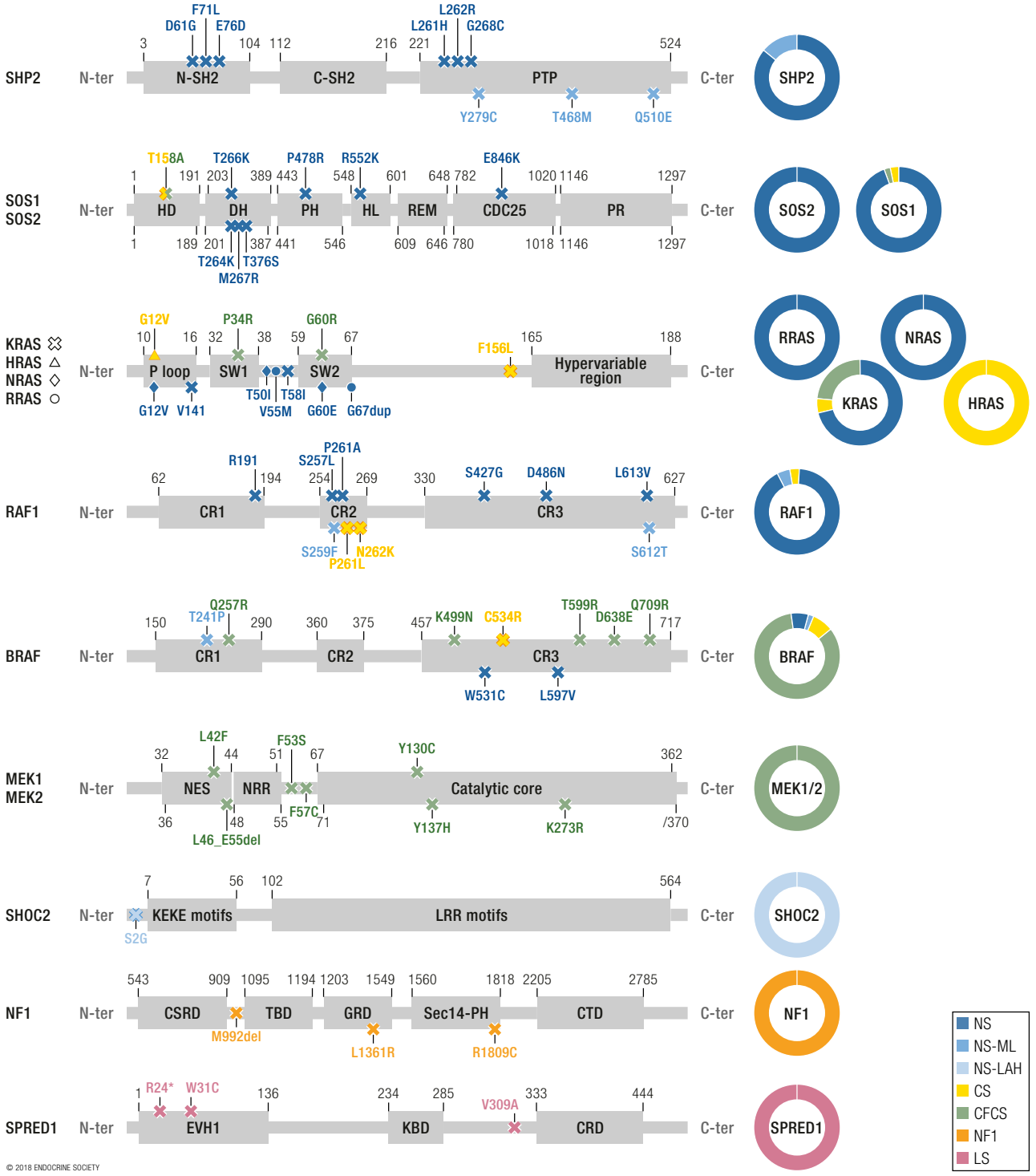
Mutations of *MEK1* and *MEK2* hit the negative regulatory region or the catalytic core domain of the kinases, resulting in increased kinase activity and gain-of-function effect on the RAS/MAPK pathway (106, 138, 160, 198, 200).

The NS-LAH–associated *SHOC2* mutation (S) has been found to create a myristoylation site, thereby resulting in constitutive translocation of SHOC2 to specific domains in the plasma membrane and prolonged PP1C activation. Subsequent BRAF/RAF1 dephosphorylation promotes sustained MAPK activation (107, 201). Mutations of *PPP1CB* encoding one of the catalytic subunits of PP1C are thought to directly or indirectly enhance PP1C phosphatase activity, leading to RAF/MEK/ERK hyperactivation, although this has not been demonstrated yet (41).

The mutations identified in the *NF1*, *SPRED1*, and *SPRY* genes mostly give rise to truncated, nonfunctional proteins [reviewed in (44, 45, 196)]. Some specific NF1 mutants (*e.g.,* those affecting Arg1809) are associated with NS features (202). NS-causing *RASA2* mutations affect two different conserved residues that are thought to alter GAP activity (203). *CBL* missense mutations impair the E3 ubiquitin ligase activity of the protein or its stability (104). Most of these mutations have been found to increase MAPK signaling through different mechanisms (*e.g.,* reduction of the GAP activity of NF1 or RASA2 and maintenance of RAS-GTP, decreased receptor turnover for CBL mutations, or loss of the SPROUTY inhibitory effect).

Altogether, RASopathy-causing mutations seem to have an activating effect on the RAS/MAPK pathway, but many modulatory events have to be taken into account. First, most of the mutations have complex and subtle consequences that result in a more qualitative than quantitative dysfunction of the RAS/

Figure 2. Structure of components of the RAS/MAPK pathway and RASopathy-causing mutations. The functional domains of the main proteins mutated in the different RASopathies are depicted, highlighting the major RASopathy-causing mutations (left panel). For each protein, the proportion of mutations associated with the different syndromes is shown [right panel; blue, NS and NS-like syndromes (NS-ML, NS-LAH); yellow, CS; green, CFCS; orange, NF1; pink, LS]. CDC25, CDC25 homology domain; CRD, cysteine -rich domain; CSRD, cysteine/serine–rich domain; CTD, carboxy-terminal domain; DH, Dbl homology domain; EVH1, enabled/vasodilator–stimulated phosphoprotein homology-1; GRD, GAP-related domain; HD, histone-like domain; HL, helical linker; KBD, c-Kit–binding domain; KEKE motif, region enriched in alternating lysine and glutamate/aspartate residues; LRR, leucine-rich region; NES, nuclear export signal; NRR, negative regulatory region; PH, Pleckstrin homology domain; P loop, phosphate-binding loop; PR, proline-rich region; PTP, protein-tyrosine-phosphatase domain; REM, Ras exchanger motif; Sec14-PH, homologous to the lipid-binding domain of the *Saccharomyces cerevisiae* phosphatidylinositol transfer protein Sec14p-pleckstrin homology domain; SH2, Src homology 2 domain; SW1 and SW2, switch region 1 and 2; TBD, tubulin-binding domain.



MAPK pathway. In favor of a qualitative effect, although copy number variants encompassing RAS/MAPK genes have been proposed as a cause of RASopathies, they often do not phenocopy syndromes caused by missense mutations (204). Second, the spatiotemporal level of RAS/MAPK hyperactivation can greatly vary depending on the mutated actor (activator, inhibitor, or central component of the pathway). Third, a large combinatorics of events can enhance or reduce the effect of a given mutation: expression-level or cell/tissue specificity of mutated protein or cofactors, stimulus specificity, and mobilization of additional signaling pathways. In the next part, we show that these events determine the penetrance/expressivity of the mutations, explaining that some tolerant cells/tissues maintain a normal function, whereas other turn into pathological dysfunctions.

Consequences of germline hyperactivation of the RAS/MAPK pathway

Cardiac defects

Considerable work has been performed to delineate the mechanisms underlying cardiac defects in RASopathies. Thus, careful analysis of the cardiac defects in zebrafish embryos indicated that expression of NS-associated as well as NS-ML–associated SHP2 variants induced loss of asymmetry, which resulted from impaired cilia function in the Kupffer vesicle, a phenotype that was corrected by pharmacological MEK inhibition (130). Cardiac defects have also been causally linked to RAS/MAPK hyperactivation in several mouse models of NS (143, 146, 147, 150, 205). Expression of NS-associated SHP2 mutants in fetal, but not adult, cardiomyocytes promotes ERK1/2 hyperactivation and is associated with ventricular and valvular abnormalities (143, 205, 206). However, comparison of tissue-specific knockin (KI) mice revealed that endothelial/endocardial–restricted expression of NS-associated SHP2 or SOS1 mutants was sufficient to trigger cardiac valve defects in NS (143, 146, 147). These apparent discrepancies may have an explanation in the time window during which the conditional expression occurs, as well as in the dysregulated communication between both tissues, in particular implying proinflammatory cytokines (TNFα, IL6), as recently suggested by Yin *et al.* (149). In $Nf1^{-/-}$ mice, defects in endothelial cushions resulting in obstructed blood flow were dependent on RAS/MAPK hyperactivation (207, 208). Cardiac defects driven by the CFCS-causing BRAF mutants could also be rescued by prenatal MEK inhibition, alone or in combination with other inhibitors (159, 209).

The pathophysiology of HCM, occurring in ~30% of affected patients and being evolutive throughout life, has been extensively studied in several models, leading to contradictory results. Two elegant studies using tissue-specific KI models of the NS-causing RAF-L613V and NS-ML–causing SHP2-Y279C mutants highlighted the relative contribution of the different cardiac cell types and of their communication in HCM development. Interestingly, both studies revealed that endothelial/endocardial expression of the mutants promoted cardiomyocyte hypertrophy (149, 152). For the two HCM-prone NS mouse models ($Sos1^{E846K}$ and $Raf1^{L613V}$), rescue experiments revealed a key role of MAPK hyperactivation in HCM development (147, 148). Distinct mechanisms have been proposed for NS-ML–associated HCM ($Ptpn11^{Y279C/+}$, $Ptpn11^{Q510E}$) (see later section), but the role of RAS/MAPK hyperactivation has not been firmly excluded.

Craniofacial defects, growth retardation, and decreased bone mass

Craniofacial defects (underlying facial dysmorphic features in humans) and growth retardation have been documented in several animal models of RASopathies (131, 143, 147, 148, 150, 159, 205). A mouse with neural crest cell–specific expression of the SHP2-Q79R, NS-causing, mutant recapitulated these skull anomalies and revealed delayed ossification of frontal bones and lack of osteogenic differentiation (210). Prenatal treatment with MEK inhibitors was necessary and was sufficient to rescue craniofacial defects, highlighting an effect of RASopathy-associated RAS/MAPK hyperactivation in skull formation during the developmental stage (150).

The mechanisms underlying growth retardation were explored in $Ptpn11^{D61G/+}$ mice. Growth retardation develops after birth and comes along with reduced IGF-1 levels and GH-evoked RAS/MAPK hyperactivation. Importantly, early treatment with a MEK inhibitor normalized IGF-1 levels and improved the growth of NS mice, implying that hyperactivation of the RAS/MAPK pathway is a driving force for this symptom (79). In addition to endocrine defects, a direct effect (IGF-I independent) of RAS/MAPK hyperactivation at the growth plate level certainly contributes to growth retardation. Indeed, very recently, NS-associated SHP2 mutants were shown to impair chondrocyte differentiation *in vitro* and *in vivo* during endochondral ossification, resulting in impaired growth plate development and reduced growth of long bones. Noticeably, this defect was rescued by MEK inhibition but not by IGF-1 treatment, although both alleviated growth retardation (211). Regarding decreased bone mass, mice with specific *Nf1* invalidation in bone and/or cartilage progenitors display enhanced osteoclastogenesis, impaired osteoblast differentiation, and defective bone mineralization, which are reverted by chronic inhibition of the RAS/MAPK pathway (165, 212–216).

Cognitive deficits

Animal models have also been valuable in deciphering the mechanisms underlying RASopathy-associated cognitive impairment. The behavioral phenotypes

of models of NF1, NS, and LS, notably learning and memory deficits and defective synaptic plasticity, were linked to hippocampal dysfunction, notably altered long-term memory induction, and to defects in neural progenitor specification and were associated with ERK1/2 hyperactivation. Consistently, treatment aimed at reducing RAS/MAPK signaling restored the cognitive defects (113, 217–221). In contrast, although CS mice also displayed enhanced ERK signaling in the brain, their cognitive deficits were not alleviated by RAS/MAPK inhibition (189). Differences in treatment protocols or in quantitative or qualitative RAS/MAPK dysregulation or involvement of alternative or compensatory mechanisms may explain these differences. Supporting this hypothesis, such treatments aimed at improving the cognitive function of patients with NF1 have given rise to divergent results (222–224). Moreover, Altmüller *et al.* (144) recently highlighted the importance of proper ERK dynamics and the existence of additional mechanisms.

Cancer predisposition

Understanding the pathophysiology of RASopathy-driven tumorigenesis is a major challenge ahead, for which animal models as well as state-of-the-art cellular tools have provided key insights. Although the contribution of RAS/MAPK hyperactivation in tumor development is quite intuitive, given its pro-oncogene function, additional mechanisms may be involved. Indeed, RAS/MAPK hyperactivation has been measured in hematopoietic cells expressing NS/JMML–associated SHP2 mutants, and this dysregulation contributes to leukemogenesis; however, other mechanisms have been identified (171, 225). In the $Kras^{V14I/+}$ mouse model of NS, chronic MEK inhibition has no effect on myeloproliferative disorder (MPD), whereas it efficiently alleviates other traits of the disease, suggesting that RAS/MAPK hyperactivation is not the main leukemogenic force (150). In contrast, similar treatment abrogated MPD and other tumor growth in mouse models of NF1 (168, 226, 227). In one CS mouse model, RAS/MAPK inhibition efficiently alleviated papilloma development (156).

Other mechanisms in RASopathy physiopathology

RAS/MAPK "independent" mechanisms

In addition to RAS/MAPK hyperactivation, several signaling pathways have been found to be dysregulated in RASopathies and to participate in their pathophysiology. Thus, a hyperactivation of PI3K/AKT signaling has been reported in models of NS-ML (151, 175, 176, 228), *SOS*-associated and *HRAS*-associated CS/CFCS (187, 229, 230), and *KRAS*-related NS/CFCS (40, 149, 187, 191, 229, 231). From a functional point of view, in several cellular and animal models of NS-ML, cardiomyocyte hypertrophy was associated with PI3K/AKT/mTOR hyperactivation and HCM was reverted by treatment with rapamycin or AKT inhibitor as well as *AKT1* genetic invalidation (151, 153, 175, 228, 232). Of note, a recent study revealed that cardiac function in an infant with NS-ML and rapidly progressive HCM was improved by 12-week everolimus (a rapamycin analogue) treatment. Although cardiac hypertrophy was not reverted in this time frame, this report provides insights into new therapies to alleviate NS-ML–associated HCM (233). The fact that hyperactivation of both RAS/MAPK and PI3K/AKT pathways give rise to similar HCM-triggering cellular defects is somewhat puzzling. However, the possibility that dysregulation of both pathways contributes jointly to HCM pathophysiology has not been addressed in the same model, although crosstalks between these two pathways are well established (234).

In addition to PI3K dysregulation, CS-associated cardiomyopathies have been causally linked to upregulation of the renin-angiotensin II system, driving a hypertensive phenotype and pointing to angiotensin convertase inhibitors as potent therapies (155).

Further highlighting a wider role for PI3K dysregulation in RASopathy pathophysiology, Kamiya *et al.* (235) recently demonstrated that targeted invalidation of *Nf1* in osteocytes resulted in increased FGF23 levels associated with an osteomalacialike bone phenotype, which was reverted by treatment with PI3K inhibitors. PI3K/AKT hyperactivation has also been measured in hematopoietic cells expressing NS/JMML–associated SHP2 mutants (171, 225). PI3K/AKT/mTOR hyperactivation is also thought to underlie the pathogenesis of multiple lentigines in NS-ML (236).

Regarding MPD and cancer development, STAT3 hypophosphorylation has been proposed as contributing to leukemogenesis in the context of NS/JMML–associated SHP2 mutants (171, 225). Enhanced dephosphorylation of STAT3 was also recently described in a mouse with pan-neuronal expression of the SHP2 E76K mutant, resulting in hydrocephalus development (237). Interestingly, using iPSC from patients with NS who did or did not develop JMML, Mulero-Navarro *et al.* (238) identified STAT5 hyperactivation as a leukemogenic signature that is associated with upregulation of specific miRNA. Moreover, NS-associated SHP2 mutants have been shown to promote MPD through alterations of mitochondrial aerobic metabolism and reactive oxygen species production (239). Pathophysiological studies identified that the SHP2-D61G mutant promotes MPD by triggering hematopoietic stem cell cycling and increasing the stem cell pool, an effect that could be mediated by proinflammatory cytokines secreted by monocytes in the vicinity of hematopoietic stem cells (240, 241). Regarding NF1, studies demonstrated that development of several tumors, including astrocytomas,

glioblastomas, MPD, and JMML, depends on the loss of other tumor suppressors (242–244).

Endocrine and metabolism imbalance: causes or consequences?

In addition to established features, a recent metabolic characterization of *Ptpn11*$^{T468M/+}$ NS-ML mice revealed a complex metabolic phenotype, associating defective adipogenesis, increased EE, and mitochondria activity/biogenesis as well as improved insulin sensitivity. These different metabolic anomalies resulted in reduced adiposity and improved carbohydrate metabolism, which could be respectively reverted by MEK inhibitor and rapamycin treatment (40). Moreover, neuronal KI of the hyperactive SHP2-D61A mutant resulted in resistance to obesity and increased EE, a phenotype that seems restricted to females because of the synergistic action of estrogens (70). This metabolic phenotype is also thought to be linked to the fact that expression of NS-associated SHP2 mutants is correlated with mitochondrial dysfunction and that patients with CS display increased EE (43, 245), suggesting that metabolic defects may be a common trait of RASopathies. In light of the complex roles of the RAS/MAPK pathway in homeostasis maintenance, one may expect that other dysfunctions of endocrine/metabolic processes will be identified. Moreover, an interesting area of research would be to determine whether such metabolic defects participate in the development of other traits of the diseases. For instance, decreased adiposity or lipodystrophy, as well as imbalance in carbohydrate metabolism or resistance to metabolic hormones (*e.g.,* insulin, leptin), have been associated with the development or worsening of several cardiopathies, notably HCM (246).

Therapeutic Perspectives

Because genetic diseases by definition affect few individuals, they have long been neglected in pathophysiological research, thereby lacking etiologic treatment. As with other rare diseases, the sole option for patients with RASopathies has long been symptomatic therapies, notably for cardiopathies and growth delay. However, their efficiency and safety are debated, and the identification of specific mutations and pathogenic mechanisms now provides new insights into specific therapies. In particular, with RAS/MAPK hyperactivation as a shared foundation, future strategies for treatment of RASopathies can thrive on therapeutic applications in the field of RAS-driven cancers. Moreover, understanding the long-term, systemic consequences of RASopathy-associated mutations will certainly shed light on additional processes to target for alleviating specific traits.

Symptomatic approaches

Similar to patients with nonsyndromic congenital heart defects, patients with RASopathies often undergo cardiac surgery, including corrective surgery for valvular malformations (valvuloplasty or primary surgical repair), ventricular septal myectomy for hypertrophy, or even heart transplantation (5). For HCM evolving to congestive heart failure, beneficial effects of beta-blocker therapy have been observed on diastolic function and ventricular remodeling (247). HCM frequently coexists with structural malformations in patients with NS, although they rarely occur in nonsyndromic HCM, which may explain worse late survival in patients with NS (248).

As a symptomatic approach for NS-associated growth retardation and before some pathophysiological mechanisms were identified, treatment with recombinant human growth hormone (rhGH) was approved by the US Food and Drug Administration in 2007 (contrary to the European Medicines Agency); however, its efficiency is still debated. Although most studies reported an enhancement of growth during the first years of treatment, the benefit of long-term therapy is uncertain. To date, only six studies reported adult height or near-adult height, with a height gain SDS varying from 0.6 to 1.4 (~4 to 11 cm, respectively). Better responses were observed with earlier initiation and longer duration of rhGH treatment (17, 249–253).

"In patients who have Costello syndrome facial features are coarser than in patients with other RASopathies."

Results of clinical trials are difficult to compare because of differing protocols (variable enrollment ages, treatment durations, and doses) and outcome criteria. None of these studies was a randomized controlled trial, and major biases affected all of the studies (254). Concerning genotype-phenotype correlations, a lower growth response was initially suggested in patients with NS and a *PTPN11* mutation (255), but it was not confirmed in long-term studies with a similar height gain compared with that of other genotypes (250). However, patients with *PTPN11* mutations presented with more severe short stature and therefore reached a lower adult height. These results may be related to GH insensitivity and/or alteration of the growth plate described in the previous section. Concerns also exist about the use of rhGH treatment in patients with RASopathies according to their tumor predisposition, notably for patients with CS, although GH deficiency has been reported for few cases (256). Regarding cardiopathies, although it was initially postulated that rhGH treatment may have an effect on ventricular development in patients with NS and heart defects, two prospective studies did not find any cause for concern (257, 258).

Targeted approaches

Given the role of RAS/MAPK hyperactivation in the pathophysiology of RASopathies, therapeutic strategies aimed at reducing this activation seem very

promising. As mentioned before, preclinical studies have demonstrated the potency of pharmacological MEK inhibitors (*e.g.,* U0126, PD0359201) in alleviating several traits of the diseases, including craniofacial defects, growth retardation, cardiopathies, and cognitive deficits (79, 143, 145, 146, 148, 220). Interestingly, because RAS/MAPK dysregulation has also been causally linked to numerous malignancies in humans, one may wonder whether RAS-targeting anticancer therapies can be repurposed for the treatment of RASopathies, at least for noncongenital features (42). Several agents targeting the RAS/MAPK pathway (*e.g.,* farnesyltransferase inhibitors, RAS antagonists, RAF, BRAF, and MEK inhibitors) are currently undergoing clinical trials in the context of cancer. However, substantial side effects and resistance mechanisms for some of them (*e.g.,* RAF inhibitors), arising from relief of negative feedback loops and requiring combinatory therapies, have been reported (47, 259). These effects may impede their transposition to the field of RASopathies, in particular when long-lasting, chronic treatment is necessary. Arguing against this notion, the therapeutic dose for treatment of RASopathies, aimed at normalizing RAS/MAPK activation, would certainly be far below cytotoxic anticancer therapies, thereby limiting both adverse effects and compensatory mechanisms.

A key difference between RASopathies and RAS-driven cancers that could influence therapeutic options is the very nature (germline vs somatic) of the mutations. Indeed, as in any genetic disease, RASopathy-associated germline mutations generate a homeostatic load, notably a sustained RAS/MAPK tonus, as soon as the earliest stage of organismal development, resulting in setting up of specific counter-regulations. Following this logic, targeting the mechanisms (*e.g.,* metabolic, endocrine, structural changes) by which the organism adapts to RAS/MAPK hyperactivation would be more potent than targeting RAS/MAPK itself.

Another limitation for the use of RAS/MAPK–targeting anticancer therapies is that specific mutations could positively, but also negatively, modify the sensitivity of the mutated protein to certain pharmacological inhibitors. Indeed, it was recently demonstrated that several NS-causing SHP2 mutants are unfortunately resistant to the SHP2 allosteric inhibitor SHP099, although this molecule displayed high efficiency, good tolerance, and oral bioavailability (260). Similarly, specific mutations of RAF1 or MEK1 have been shown to make mutated derivatives resistant to inhibitors targeting the corresponding wildtype kinases (261, 262).

Beyond the parallel between RASopathies and RAS-driven cancers, the use of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, also known as "statins," has been suggested as a potential therapy for RASopathies. Indeed, it has been proposed that statins can decrease RAS activity by reducing RAS farnesylation and its localization to the plasma membrane (263). Although this effect may not be restricted to RAS but affects all farnesylated proteins in a nonspecific manner, a fine-tuned dosage may normalize RAS activation without affecting other functions in the context of RAS hyperactivation. Thus, the efficacy of statins for the treatment of cognitive deficits has been reported in mouse models of NS and NF1 (218, 220). Statins (*e.g.,* simvastatin) have also been assessed in clinical studies for the treatment of cognitive deficits in children with NF1 with no significant or minor effects but good tolerance (222, 223). Moreover, it was recently reported that statin treatment may improve growth and correct impaired chondrocyte differentiation in an NS mouse model (211). Clinical trials are needed to test whether statin treatment could be a therapeutic strategy to alleviate growth retardation and other noncongenital features in patients with NS. A phase 3, randomized, double-blind, placebo-controlled therapeutic trial is under way to test the efficiency and safety of simvastatin to treat growth and bone abnormalities in children with NS (ClinicalTrials.gov identifier: NCT02713945).

In addition to inhibition of the RAS/MAPK pathway, specific alterations could be targeted. In the case of NS-ML, which has been causally linked to PI3K/AKT/mTOR hyperactivation, pharmacological strategies aimed at inhibiting this pathway (rapamycin, AKT inhibitor) have efficiently alleviated some symptoms of the disease in mouse models and also in patients (151, 228, 233). In patients with NS with activating *PTPN11* mutations, another approach will be the pharmacological inhibition of SHP2 activity, as such molecules have been developed in the cancer field and tested in mouse tumor models (264). In addition, several studies have suggested the contribution of proinflammatory cytokine signaling in RASopathy pathophysiology, notably in HCM development, as treatment with cyclosporine or TGFβ inhibitors has reversed cardiomyocyte hypertrophy (265, 266). Moreover, current efforts are directed toward the screening of new molecules that could revert specific RASopathy-associated phenotypes in cellular (*e.g.,* iPSC-derived engineered cardiac tissues) and animal models (*C. elegans*, zebrafish) (267).

Future Directions

Within less than 30 years, considerable advances have been made in the understanding of RASopathy pathophysiology, with the identification of causal mutations and the functional analysis of their pathophysiological consequences. However, many questions remain unanswered. Indeed, there are still

unidentified causal genes for a large proportion of patients diagnosed with RASopathies. For those new genes, as well as for recently identified mutations, functional analyses will enrich our understanding of RASopathies. Future work will also be directed toward identifying the precise mechanisms underlying the similarities and differences between RASopathies and the variable expression of a given mutation. Moreover, although several molecular defects have been identified, notably for major congenital features, they often rely on organ/function–directed approaches, so we still have a fragmentary view of the global pathophysiology.

Major challenges ahead are thus to delineate the integrated effect of RASopathy-associated mutations, taking into account recently identified endocrine and metabolic imbalances and interorgan interactions, and to assess the relative contribution of the different alterations to the global phenotype. The likely contribution of both autonomous and non-autonomous mechanisms in any investigated function is certainly an important obstacle, which may be overcome by the combined use of multiple tissue-specific and inducible models, as well as multiscale differential studies, modeling, and computation analysis. Moreover, additional levels of complexity will certainly emerge, including genetic, environmental, or age-related and sex-related modifiers. Longitudinal clinical studies on bigger cohorts of patients will certainly provide important insights about such contributions and will allow robust genotype/phenotype correlations. All this knowledge to come will allow the development of personalized medicine aimed at normalizing the more relevant pathogenic mechanisms. Beyond rare diseases, understanding of the consequences of germline activation of RAS/MAPK can have a wider fallout in the field of chronic disorders.

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Abbreviations

CFCS, cardio-facio-cutaneous syndrome; CR, conserved region; CS, Costello syndrome; EE, energy expenditure; ERK, MAPK extracellular signal-regulated kinases; GAP, GTPase-activating protein; GTP, guanosine triphosphate; HCM, hypertrophic cardiomyopathy; iPSC, induced pluripotent stem cell; JMML, juvenile myelomonocytic leukemia; KI, knockin; LOF, loss-of-function; LS, Legius syndrome; MEK, MAPK ERK kinases; MIM, Mendelian Inheritance in Men; MPD, myeloproliferative disorder; NF1, type I neurofibromatosis; NS, Noonan syndrome; NS-LAH, Noonan-like syndrome with loose anagen hair; NS-ML, Noonan syndrome with multiple lentigines; PI3K, phosphatidylinositol 3-kinase; PTP, protein tyrosine phosphatase; *PTPN11*, protein tyrosine phosphatase non-receptor type 11; PVS, pulmonary valve stenosis; rhGH, recombinant human growth hormone; SDS, standard deviation score.