

# Expansion of the RASopathies

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## Abstract

**Purpose of Review** The Ras/mitogen-activated protein kinase (MAPK) pathway is essential in the regulation of cell cycle, differentiation, growth, cell senescence and apoptosis, all of which are critical to normal development. A class of neurodevelopmental disorders, RASopathies, is caused by germline mutations in genes of the Ras/MAPK pathway. Through the use of whole exome sequencing and targeted sequencing of selected genes in cohorts of panel-negative RASopathy patients, several new genes have been identified.

**Recent Findings** New genes have been identified and include *RIT1*, *SOS2*, *RASA2*, *RRAS* and *SYNGAP1*, that likely represent new, albeit rare, causative RASopathy genes. In addition, *A2ML1*, *LZTR1*, *MYST4*, *SPRY1* and *MAP3K8* may represent new rare genes for RASopathies, but, additional functional studies regarding the mutations are warranted. In addition, recent reports have demonstrated that chromosomal copy number variation in regions encompassing Ras/MAPK pathway genes may be a novel pathogenetic mechanism expanding the RASopathies.

**Summary** The identification of potential new genes and chromosomal copy number variation being associated with

the RASopathies is very exciting and broadens our understanding of the biology of Ras signaling and the RASopathies.

**Keywords** Noonan syndrome · RASopathy · Ras/MAPK pathway · *RIT1* · Signal transduction · *SYNGAP* · *MAP3K8*

## Introduction

### The RASopathies

The RASopathies are a class of developmental disorders that are caused by germline mutations in genes which encode components of the Ras/mitogen-activated protein kinase (MAPK) pathway [1•]. This includes the core pathway components and both positive and negative regulators of Ras signaling. The Ras/MAPK pathway is a ubiquitous, highly conserved, intracellular signaling pathway that is critical in cell cycle regulation, differentiation, growth, apoptosis, and cell senescence. It plays an essential role throughout mammalian development, from embryogenesis to tissue-specific cellular homeostasis in the adult. Therefore, it is not surprising germline mutations that affect its function can have a profound deleterious effect on development. Taken together, the RASopathies represent one of the most prevalent groups of congenital malformation syndromes affecting approximately 1 in 1000 individuals. These syndromes include (1) neurofibromatosis type 1 (NF1), caused by loss of function mutations in the protein neurofibromin [2–4]; (2) Noonan syndrome (NS) caused by mutations in *PTPN11* [5], *SOS1* [6, 7], *RAF1* [8, 9], *KRAS* [10], *NRAS* [11], *SHOC2* [12], and *CBL* [13, 14]; (3) NS with multiple lentiginos caused by

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mutations in *PTPN11* [15] and *RAF1* [9]; (4) Legius syndrome caused by inactivating mutations in the *SPRED1* gene [16]; (5) Costello syndrome (CS) caused by activating mutations in *HRAS* [17]; (6) cardio-facio-cutaneous syndrome (CFC) caused by gain of function mutations in *BRAF* [18, 19] and *MAP2K1* or *MAP2K2* [19]; and (7) capillary malformation-arteriovenous malformation (CM-AVM) caused by loss of function mutations in the *RASA1* gene [20]. The RASopathies share many overlapping characteristics including craniofacial dysmorphism; cardiac, cutaneous, skeletal, and ocular abnormalities; neurocognitive impairment; hypotonia and impaired growth; and, in many, an increased cancer risk, because they all share the common pathogenetic mechanism of Ras/MAPK pathway signaling dysregulation and/or activation. However, because each of the syndromes is due to mutations in specific affected genes of the pathway, and distinct mutations within that gene, each has unique phenotypic features that result from the specific fashion by which the Ras/MAPK pathway is dysregulated.

### Ras Signaling

The Ras proteins are small guanosine nucleotide-bound GTPases that act as a central signaling hub for multiple intracellular signaling pathways. Ras genes exist as a multigene family that includes *HRAS*, *NRAS*, and *KRAS*. Ras proteins cycle between an active guanosine triphosphate (GTP)-bound form and an inactive guanosine diphosphate (GDP)-bound form. Ras signals through two well-characterized effector pathways, the Ras/MAPK (Raf–MEK–ERK) and the phosphatidylinositol-3-kinase (PI3K) pathway, as well as through numerous other effector pathways including RalGDS, TIAM, PLC $\epsilon$ , and AF6 [21]. Functional studies of mutant proteins associated with the RASopathies indicate that the common pathogenetic mechanism is activation and dysregulation of the Ras/MAPK pathway [1•]. Activation of Ras begins with the binding of growth factors to receptor tyrosine kinases (RTKs), G-protein coupled receptors, or other receptors that result in the recruitment of guanine-nucleotide exchange factor (GEF) son of sevenless (SOS) to the membrane. At rest, SOS is complexed with the adapter protein GRB2 (growth factor receptor-bound protein 2). Recruitment is mediated by the SH2 domain of GRB2 which interacts with the activated receptor tyrosine kinase (RTK). This brings SOS into the vicinity of Ras leading to the exchange of GDP for GTP, thereby switching Ras into the active state. Although Ras proteins have an intrinsic GTPase activity, it is too low to effectively inactivate Ras. Therefore, the conversion to the inactive state requires proteins that accelerate GTP hydrolysis, and GTPase-activating proteins (GAPs) such as neurofibromin or RASA1.

Activated Ras leads to the activation of Raf proteins. Raf phosphorylates and activates MEK1/MEK2 (MAPK kinase) which then phosphorylates and activates the terminal MAPKs of the pathway ERK1 and/or ERK2. ERK1/2 function on a large number of downstream molecules, both nuclear and cytosolic. ERK1/2 substrates include transcription factors, membrane proteins, and protein kinases that, in turn, control essential cellular functions [22].

Although activation of the Ras/MAPK pathway has been shown to be the predominant biochemical hallmark of the RASopathies, aberrant Ras signaling through other effector pathways is likely to also play a significant role [23, 24]. In addition, the evolution of our understanding of the complexity of Ras signaling, and the increased availability of new powerful tools of discovery, such as exome/genome sequencing, will inevitably lead to the expansion of the pathogenetic etiology of the RASopathies. Numerous patients have phenotypic features suggestive of a RASopathy; however, they have no mutations in any of the known RASopathy causative genes. For example, the pathogenetic cause of approximately 20 % of patients with the clinical diagnosis of RASopathy or a RASopathy-like clinical phenotype remains unknown. In this review, we highlight recent studies that have identified a number of new genes and potential new genes associated with the RASopathies (Table 1).

### New Genes Associated with RASopathies

#### RIT1

Aoki et al. examined a NS cohort of 180 individuals with no known NS mutations [25]. They identified 17 individuals with nine unique missense mutations in *RIT1* by whole exome sequencing and targeted sequencing of *RIT1*. Ras-like protein in tissues (RIT1) is a member of a novel branch of Ras-related GTPase proteins in the Ras family. Other members in the Ras subfamily include Ras-like protein in neurons (RIN) and Ras-like protein that interacts with calmodulin (RIC) [26]. All share approximately 50 % structural homology with Ras but lack a C-terminal lipidation site. The nine missense mutations include c.104G > C encoding p.S35T; c.176C > G encoding p.A57G; c.242A > G encoding p.Q81G; c.244T > G encoding p.F82 V; c.246T > G encoding p.F82L; c.247A > C encoding p.T83P; c.265T > C encoding p.Y89H; c.270G > T encoding p.M90I; and c.284G > C encoding p.G95A. Many of these RIT1 mutants and two additional missense mutants (p.A77G and p.A77P) were identified in follow-up studies in other NS cohorts with no known NS causing gene mutations [27, 28, 29•]. Most of

**Table 1** New genes associated with RASopathies

Gene	Protein name	Protein function	Chromosome location	Pathogenetic mechanism	References
<i>MYST4</i>	Histone acetyltransferase (monocytic leukemia-4)	Histone acetyltransferase	10q22.2	Loss of function mutation; haploinsufficiency	Kraft et al. [51]
<i>RIT1</i>	Ras-like protein in tissues	RasGTPase	1q22	Activating mutation	Aoki et al. [25]
<i>RASA2</i>	RAS p21 protein activator 2	RasGAP	3q23	Loss of function mutation; haploinsufficiency	Chen et al. [29•, 56]
<i>RRAS</i>	Related ras viral (R-Ras) oncogene homologue	RasGTPase	19q13.33	Activating mutation	Flex et al. [35]
<i>SOS2</i>	Son of sevenless homologue 2	RasGEF	14q21.3	Activating mutation	Yamamoto et al. [31]
<i>LZTR1</i>	Leucine-zipper-like transcriptional regulator 1	Substrate adaptor protein	22q11.21	Loss of function mutation; haploinsufficiency	Vissers et al., 2015
<i>A2ML1</i>	Alpha-2-macroglobulin-like 1	Secreted broad-range protease inhibitor	12p13.31	Loss of function mutation; haploinsufficiency	Yamamoto et al. [42]
<i>SYNGAP1</i>	Synaptic ras GTPase-activating protein 1	RasGAP neuronal specific	6p21.3	Loss of function mutation; haploinsufficiency	Hamdan et al. [38]
<i>Spry1</i>	Sprouty1	Negative regulator Ras/MAPK pathway	4q28.1	Loss of function mutation; haploinsufficiency	Chen et al. [29•, 56]
<i>MAP3K8</i>	Mitogen-activated protein kinase kinase kinase 8	Serine/threonine protein kinase family activates MAPK and JNK pathways	10p11.23	Activating mutation	Chen et al. [29•, 56]

the RIT1 mutations are in the switch I or II regions and are predicted to result in a constitutively active Ras protein. RIT1 has been shown to activate the Ras/MAPK pathway [30]. Functional analysis of *RIT1* mutations by transient transfection into NIH3T3 and HEK293 cells demonstrated increased signaling of the Ras/MAPK pathway [25, 29•].

## SOS2

Yamamoto et al. in 2015 identified two de novo heterozygous missense-causing mutations in *SOS2* by whole exome sequencing in a cohort of 58 probands with the clinical diagnosis of NS, but who were negative for known NS mutations [31]. *SOS2* encodes the protein son of sevenless 2, a RasGEF and homologue to *SOS1*, the second most frequently mutated gene in NS. The *SOS2* mutations identified, p.M267K and p.T376S, are both located in the Dbl homology (DH) domain responsible for maintaining *SOS2* in an autoinhibited conformation. In a subsequent study, targeted sequencing of *SOS2* was carried out in an additional cohort of 150 clinically diagnosed NS patients also negative for known NS mutations [32]. They confirmed the *SOS2* p.T376S missense mutation in four

individuals, one of which was maternally inherited. In addition, they identified two novel de novo missense mutations: p.M267R and p.T264K. The p.M267 location in *SOS2* is homologous to the p.M269 location in *SOS1*, a common mutation site in NS [31]. *SOS2* mutants p.T264K, p.M267R, and p.T376S were functionally assessed in vitro using HEK293 cells. All three mutants resulted in higher levels of GTP-bound Ras and increased signaling of the Ras/MAPK pathway consistent with the known mechanism of the NS causative *SOS1* mutations [32].

## RASA2

In order to identify additional genes that may be associated with NS, Chen et al. [29•] performed whole exome sequencing on a cohort of 27 patients with a NS clinical diagnosis that did not have any mutations in known NS causing genes. They identified three patients with novel missense-causing mutations in the *RASA2* gene. *RASA2* encodes the RasGAP protein Ras P21 protein activator 2, *RASA2*. The three mutations identified affected two different amino acid residues p.Y326 and p.R511 of which both are in the conserved GAP domain (p.Y326C,

p.Y326N, and p.R511C). The p.R511C substitution is in the Ras interaction site and is predicted to act as a dominant-negative competitor for Ras binding. Therefore, the mutations are assumed to result in loss of function. Overexpression of RASA2 mutant proteins in HEK293 cells resulted in prolonged Ras/MAPK signaling following epidermal growth factor stimulation. Loss of function mutations in RasGAPs are responsible for other RASopathies including NF1 caused by mutations in *NF1* [2, 3, 33] and CM-AVM caused by mutations in *RASA1* which encodes p120 RasGAP1 [20]. Moreover, missense mutations in RASA2 have been identified in human melanomas in addition to other cancers [34].

## RRAS

Flex et al. in 2014 [35] sought to identify additional RASopathy-associated genes by screening for mutations in a selected group of candidate genes based on their predicted protein interaction/function with known RASopathy-associated proteins. In a RASopathy cohort of 96 unrelated individuals with no known RASopathy-associated mutations, they identified one missense-causing mutation in the *RRAS* gene in a patient described as having some phenotypic features of NS. The mutation, c.163G > A, results in a p.V55M residue substitution. It was not established if this mutation was de novo since the parents were not examined. This mutation was reported to occur in 1/13,000 alleles by the NHLBI exome sequencing project. The *RRAS* gene encodes RRAS (related ras viral (R-Ras) oncogene homologue) that exhibits 50–60 % homology to the Ras proteins. It is associated with several diverse cellular processes, including neuronal axon guidance, angiogenesis, cell adhesion, and migration. Activating mutations in *RRAS* are associated with numerous cancers [36]. Sequencing of *RRAS* in an additional cohort of 408 patients with a RASopathy phenotype who were also negative for known RASopathy gene mutations revealed one individual with a heterozygous 3 nucleotide duplication, c.116\_118dup resulting in p.G39dup. Functional analysis of the mutant RRAS protein showed a decrease in intrinsic GTPase activity in an in vitro assay. Overexpression of mutant proteins in COS-7 cells increased Ras/MAPK pathway signaling, consistent with other RASopathy causing gene mutations. Therefore, activating mutations in *RRAS* may represent a rare RASopathy-associated gene with a NS phenotype.

## SYNGAP1—A New RASopathy

RasGAPs (GTPase-activating proteins) are negative regulators of the Ras/MAPK pathway. Haploinsufficiency in neurofibromin (encoded by the *NF1* gene), p120 RasGAP

(encoded by the *RASA1* gene), and Ras P21 protein activator 2 (encoded by the *RASA2* gene) have all been found to cause RASopathies. SynGAP1 (synaptic Ras GTPase-activating protein 1) is a recently described RasGAP thought to be expressed only in neurons (for recent review see [37]). Germline mutations in *SYNGAP1* have been identified to cause autosomal dominant intellectual disability type 5 which is considered a nonsyndromic form of intellectual disability [38]. De novo heterozygous frame shift mutations were identified in individuals with nondysmorphic features, global delay, and hypotonia who also had moderate to severe intellectual disability. Other phenotypic features that were identified in the original cohort include seizures and ophthalmologic findings. More recently, expansion of the phenotype came to include global developmental delay with behavioral issues (aggressive behavior, sleep disturbances, and hyperexcitability) and autism spectrum disorder [39]. Mild dysmorphic features were also described and included microcephaly, myopathic facies, deep set eyes, long nose, and long ears with protuberant lobes. Additional features may include pectus, kyphosis, hip dysplasia, strabismus, fine hirsutism, and significant constipation with failure to thrive.

SYNGAP1, encoded by *SYNGAP1*, is located on chromosome 6p21.3. It is a major component of the postsynaptic density found associated with excitatory *N*-methyl-D-aspartate (NMDA) receptors at synapses. Its GAP domain is homologous to that of p120 RasGAP and neurofibromin, two RasGAPs known to be associated with RASopathies. SYNGAP1 is phosphorylated by calmodulin-dependent protein kinase II, CaMKII, causing a decrease in its RasGAP activity resulting in an activation of the Ras/MAPK pathway. Mouse models of SynGAP1 +/- mice demonstrate alteration in Ras/MAPK signaling, synaptic plasticity, and learning, and activated ERK in hippocampal extracts [40]. The behavioral phenotype of SynGAP1 +/- mice, which express roughly half the normal levels of SynGAP1, exhibits multiple behavioral traits such as aberrant cognitive and noncognitive processes that are normally mediated by the hippocampus. Mice are also hyperactive, have reduced spatial alternation, and have severe working and reference memory deficits [41].

Although patients with SYNGAP1 mutations have several phenotypic features characteristic of upregulation of the Ras/MAPK pathway, individuals with SYNGAP1 mutations have not been noted to have the classical craniofacial phenotypic features which traditionally have been associated with RASopathies. Based on the unique neurologically centered clinical phenotype, the identification of causative mutations in a new RasGAP gene with neuronal restricted expression, and the fact that it does not categorize with any established RASopathy, we consider this a new RASopathy.

## Candidate RASopathy Genes

### A2ML1

Using whole exome sequencing in both the proband and parents to detect de novo variants, Vissers et al. reported a mutation in the *A2ML1* gene in a proband with a clinical diagnosis of NS. The mutation, c.2405G > A, resulted in a p.R802H substitution [42]. *A2ML1* which encodes the protein alpha-2-macroglobulin-like 1 (A2ML1) is a secreted broad-range protease inhibitor [43]. Autoantibodies to A2ML1 have been reported to be responsible for paraneoplastic pemphigus (PNP), a multi-organ syndrome characterized by intractable stomatitis, polymorphous cutaneous lesions, and lympho-proliferative tumors [44]. However, the c.2405G > A variant was also reported in 4/12,194 alleles in the NHLBI exome sequencing project. This initial variant finding was expanded upon by targeted sequencing of *A2ML1* in a cohort of 155 NS patients with no identified RASopathy causing mutations. They identified two individuals with missense mutations in *A2ML1*: One with the same mutation they previously found, c.2405G > A encoding a p.R802H missense substitution, and a second mutation c.1775G > T encoding p.R592L. Both of these were shown to segregate with the individuals offspring who also exhibited a NS-like phenotype. The individual with the p.R802H mutation had inherited it from his mother, but surprisingly she was reported to exhibit no RASopathy phenotype. Both the mutations are predicted to impair protein function. In addition, a study examining the genotype–phenotype correlation for external ear anomalies and hearing impairment in a NS cohort identified one individual with a c.4061 + 1G > A mutation in *A2ML1* [45]. The functional consequences of the A2ML1 mutations on Ras/MAPK pathway signaling were examined by overexpression transfection experiments in HEK293T and COS7 cells. However, neither mutation resulted in increased phosphorylated ERK levels [42]. The authors theorized that one binding target of A2ML1 is lipoprotein receptor-related protein 1 (LRP1) which is an upstream activator of the Ras/MAPK pathway. LRP1 associates with SHC domain proteins and CBL during recruitment to the plasma membrane [46, 47]. These findings suggest that rare *A2ML1* mutations may be associated with a RASopathy exhibiting a highly variable NS-like phenotype; however, further studies are needed to confirm the causality.

### LZTR1

Yamamoto and colleagues identified five missense-causing variants in *LZTR1* by whole exome sequencing in a cohort of 58 probands with the clinical diagnosis of NS, but who were

negative for known NS mutations [31]. The missense mutations consisted of p.G248R p.R284C, p.R287Y, p.Y119C, and p.S247N. Of these, two of the mutants p.G248R and p.S247N were also shown to segregate with the NS phenotype within two large families. In addition, Chen and colleagues identified two heterozygous *LZTR1* missense mutations in similar regions (p.R237Q and p.A249P) by exome sequencing in their NS patient cohort [29]. However, the functional characterization of these missense mutations was not commented upon. The *LZTR1* (leucine-zipper-like transcriptional regulator 1) protein belongs to a functionally diverse family of proteins containing BTB-kelch domains that is thought to localize to the cytoplasmic surface of the Golgi membrane [48]. All of the mutations are in the highly conserved kelch domain and are predicted to disrupt protein function. Kelch domains form a tertiary structure that may also be responsible for binding to other proteins [49]. Somatic *LZTR1* mutations have been described in glioblastoma. In addition, germline *LZTR1* mutations are associated with schwannomatosis, one of the neurofibromatoses characterized by late onset tumor predisposition [50]. These findings suggest that mutations in *LZTR1* may be responsible for a rare percentage of NS cases. However, it is not known if the identified *LZTR1* mutations increase Ras/MAPK signaling, the underlying pathogenetic mechanism of the RASopathies.

### MYST4

Karyotyping of a patient with a clinical diagnosis of NS who did not have any identified mutations in genes known to be causative of NS revealed a balanced de novo chromosomal translocation t(10;13) (q22.3;q34) [51]. The translocation breakpoint was mapped to 10q22.3 to a location disrupting the *MYST4* gene causing *MYST4* haploinsufficiency. The *MYST4* gene, also known as *KAT6B*, encodes MYST histone acetyltransferase (monocytic leukemia) 4 (also termed, K(Lysine) acetyltransferase 6B). Histone acetyltransferases modify DNA by transferring an acetyl group from acetyl-CoA to histone proteins on DNA. This epigenetic modification of DNA plays an important role in gene regulation. Generally, increased histone acetylation globally results in increased transcriptional activation. The authors demonstrated in a lymphoblastoid cell line derived from the patient that haploinsufficiency of *MYST4* resulted in an increase of Ras/MAPK pathway activity. In addition, genome-wide chromosome immunoprecipitation analysis demonstrated that gene targets of *MYST4* were somewhat biased toward MAPK signaling pathways. The authors postulated that altered expression of multiple genes associated with Ras/MAPK pathway regulation may be responsible for the increase in pathway

activation and the NS-like phenotype. However, more research is necessary to confirm this novel correlation.

### SPRY1

A nonsense de novo mutation, p.E79\*, in *SPRY1* was identified by whole exome sequencing in one individual in a cohort of 27 clinically diagnosed NS patients with no known NS associated mutations [29•]. *SPRY1* encodes the protein Sprouty1 which is a negative regulator of Ras/MAPK pathway signaling. The complete mechanism by which Sprouty1 inhibits Ras/MAPK signaling remains unclear, it is thought to act at the level of the signaling from the RTK to Ras [29•]. Functional studies need to be carried out to establish whether or not this *SPRY1* nonsense mutation results in increased Ras/MAPK pathway signaling.

### MAP3K8

Chen et al. identified a de novo missense mutation p.L128V in *MAP3K8* in one individual in the 27 NS patient cohort described above. *MAP3K8* encodes mitogen-activated protein kinase kinase kinase 8 (MAP3K8) which has been shown to cause lung cancer and can directly activate MEK1 [52]. The significance of the p.L128V mutation is not known because although it is in a conserved region, the function of the region is not known. However, functional analysis by overexpression of the mutant protein in HEK293 cells resulted in increased levels of phosphorylated ERK. Additional studies in additional RASopathy cohorts will need to be carried out to confirm the pathogenetic significance of this gene.

### Copy Number Variation Associated with RASopathy Genes

Over the past several years, rare copy number variants of genes encoding Ras pathway components have been reported in individuals with phenotypic features reminiscent of RASopathy syndromes. Multiple studies have identified chromosomal duplications encompassing *PTPN11* in patients with a clinical diagnosis of NS [53–55]. *PTPN11* encodes SHP2 which facilitates Ras activation. Gain of function missense mutations in SHP2 are the most common mutations associated with NS, accounting for approximately 50 % of all cases. Chromosomal duplication of region 12q24.13 encompassing the *PTPN11* gene was detected by array comparative genomic hybridization (array CGH) [53, 54]. Moreover, a de novo trisomy of 12q24.11q24.21 encompassing the *PTPN11* gene was recently reported [55]. In each of these cases,

gene duplication is thought to result in increased dosage of SHP2 which has the equivalent effect as the common gain of function mutation in *PTPN11* associated with NS. In addition, rare chromosomal duplications associated with *SHOC2* in a NS patient [56], and *RAF1* and *MAP2K2* in patients with mild RASopathy spectrum phenotypes have been reported [57, 58]. Chromosomal deletions have also been reported to be associated with a RASopathy phenotype. A chromosomal deletion encompassing the *BRAF* locus at 7q34 was reported in two individuals with phenotypes overlapping several RASopathies [58, 59]. In addition, two studies reported interstitial deletion of 19p13.3 encompassing the *MEK2* gene [60, 61]. The patients had clinical features suggestive of dysregulation of the Ras/MAPK pathway but were not classical for CFC or any of the other RASopathies. Functional studies of the *MEK2* deletion caused *MEK2* haploinsufficiency and resulted in dysregulation of the Ras/MAPK pathway [60].

### Conclusion

The initial identification of the genes causative for RASopathies which exhibit overlapping phenotypic features, also exhibit many disparate characteristics as well, has enabled us to classify these syndromes as to their common underlying pathogenetic etiology, the dysregulation of the Ras/MAPK pathway. The RASopathies caused by germline mutations in genes encoding components of the Ras/MAPK pathway underscore the essential role the pathway plays in normal embryonic and postnatal development, since dysregulation of the pathway has severe developmental consequences. In addition, the number of different affected genes and the diversity of mutations within each gene are reflected in the variety of affected phenotypic features present in this class of syndromes. The accurate identification of a causative gene mutation for a given genetic disorder is essential in establishing a molecular diagnosis corresponding to the clinical diagnosis and enables the physician to accurately manage the health of the patient. Numerous patients have phenotypic features suggestive of a RASopathy; however, they have no mutations in any of the known RASopathy causative genes. Recent advances in genome sequencing technology provide unprecedented capabilities for the discovery of rare genes causative for genetic disorders. In particular, the modest cost of whole exome sequencing has made it increasingly useful in this endeavor. With the application of this technology, the identification of a number of genes potentially causative for RASopathies has expanded. Several of the recently identified genes associated with the RASopathies including, *RIT1*, *SOS2*, *RASA2*, *RRAS* and *SYNGAPI*, likely represent new, albeit rare, causative

RASopathy genes. They were identified in multiple patients, some segregated with the phenotype, and the function of their encoded protein clearly links them to Ras signaling. In addition, several genes were identified that are potentially associated with the RASopathies; however, their link to Ras signaling is novel, and their link to Ras signaling is not entirely understood. These include *A2ML1*, *LZTR1*, and *MYST4*. They may represent new rare genes causative for RASopathies, but additional studies regarding the function of their encoded proteins and the effects of the mutations are warranted. The identification of these genes being associated with the RASopathies is very exciting and broadens our understanding of the biology of Ras signaling and the RASopathies. Because of the increased application of genome and exome sequencing, based on next generation sequencing technology to discover new genetic causes for genetic disorders, it is challenging to determine if the detected gene variant is truly pathogenetic. Therefore, the College of Medical Genetics and Genomics and the Association for Molecular Pathology have recently addressed this challenge and have suggested standards and guidelines for interpretation of the sequence variants [62]. Utilizing this framework will help standardize the criteria for the assignment of a new gene variant as being causative for a given disorder. In addition, since the underlying biochemical phenotype associated with the RASopathies is activation of the Ras/MAPK pathway, functional studies regarding new gene variants are essential in establishing their causality.

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#### Compliance with Ethical Guidelines

**Disclosure** William E. Tidyman and Katherine A. Rauen declare that they have no conflict of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by any of the authors.

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