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## Authors
Van, Richard
Cuevas-Navarro, Antonio
Castel, Pau
et al.

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# The molecular functions of RIT1 and its contribution to human disease

**Richard Van**, **Antonio Cuevas-Navarro**, **Pau Castel**, **Frank McCormick**

Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, CA, U.S.A.

## Abstract

RIT1 is a member of the Ras family of GTPases that direct broad cellular physiological responses through tightly controlled signaling networks. The canonical Ras GTPases are well-defined regulators of the RAF/MEK/ERK pathway and mutations in these are pathogenic in cancer and a class of developmental disorders termed RASopathies. Emerging clinical evidences have now demonstrated a role for RIT1 in RASopathies, namely Noonan syndrome, and various cancers including lung adenocarcinoma and myeloid malignancies. While RIT1 has been mostly described in the context of neuronal differentiation and survival, the mechanisms underlying aberrant RIT1-mediated signaling remain elusive. Here, we will review efforts undertaken to characterize the biochemical and functional properties of the RIT1 GTPase at the molecular, cellular, and organismal level, as well as provide a phenotypic overview of different human conditions caused by RIT1 mutations. Deeper understanding of RIT1 biological function and insight to its pathogenic mechanisms are imperative to developing effective therapeutic interventions for patients with RIT1-mutant Noonan syndrome and cancer.

## Introduction

The family of Ras guanosine triphosphate hydrolases (GTPases) are widely recognized for their oncogenic potential in human cancers and have prompted intensive research efforts, revealing fundamental signaling networks and molecular mechanisms contributing to disease since their discovery in the 1980s [1,2]. Somatic mutations in the canonical *HRAS*, *NRAS*, and *KRAS* genes are observed in a large number of human cancers, including lung, pancreatic, and colorectal adenocarcinomas, as well as skin cancers and leukemias among others [3]. Furthermore, germline mutations in genes encoding components of the Ras/mitogen activated protein kinase (MAPK) pathway lead to a distinct class of developmental disorders, called RASopathies, which are characterized by difficulty to thrive, learning disabilities, dysmorphic features, cardiovascular and lymphatic anomalies, and predisposition to neoplasia [4]. The RAS family has expanded to over 150 human Ras-related GTPases, many of which are evolutionarily conserved in metazoans, and their characterizations have provided insights into fundamental cellular processes [1]. Among

**Correspondence:** Frank McCormick (Frank.mccormick@ucsf.edu).

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

these, RIT1 (Ras-like without CAAX 1) was first identified over two decades ago [5], and emerging evidence suggests that RIT1 plays a causal role in certain cancers and Noonan syndrome (NS), the most common RASopathy [6,7]. However, the molecular mechanisms and biological functions of RIT1 remain elusive, and more insight into its pathophysiological contributions are needed to create effective therapeutic interventions for patients harboring RIT1 mutations.

Ras proteins undergo conformational changes when converting between inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound states, thus enabling binding to effector proteins and switch-like activation of downstream signaling pathways. Guanine nucleotide exchange factors (GEFs) mediate the conversion of inactive GDP-bound to active GTP-bound conformations by promoting the release of GDP. This conversion is reversed by GTPase-activating proteins (GAPs) which catalyze the intrinsic GTP hydrolysis of RAS proteins [8]. Together, GEFs and GAPs provide a level of essential regulation of Ras-related GTPases to mediate a context-dependent cascade of molecular events in a timely manner (Figure 1A).

The identification of RIC (Ras-related protein interacting with calmodulin) in *Drosophila melanogaster* led to the subsequent identification of human RIT1 and RIN (also known as RIT2) [5]. RIT1 and RIN share 68% amino acid sequence similarity, while RIC shares 55% and 57% sequence similarity to RIT1 and RIN, respectively (Figure 1B). Additionally, much of the similarity between RIC and RIT is preserved in the G-domain while the amino (N)- and carboxy (C)-terminal regions are largely divergent. The *RIT1* gene is located at chromosome 1q22 and spans 13.5 kb in genomic length, containing 6 exons and 5 introns. The start codon is located in exon 2 and gives rise to the canonical RIT1 isoform 2 (NM_006912.6 and NP_008843.1). While RIT1 is ubiquitously expressed across many tissues, human gene expression data obtained from the GTEx Portal [9] indicate that *RIT1* mRNA is highly expressed in the lung, esophagus, blood, vagina, and spleen. Tissues that exhibit lower expression include brain, pancreas, liver, and skeletal muscle. In contrast, the closely related RIN GTPase appears to be only expressed in the brain (Figure 1C).

RIT1 has been mostly described as a regulator of neuronal cell proliferation, survival, and differentiation [10], yet growing clinical evidence suggests that RIT1 may play an important role in other tissues. For instance, NS patients with RIT1 mutations very often present with congenital heart defects, and neoplasms with somatic mutations in RIT1 can arise from the lung [6,7]. Moreover, high expression of RIT1 has been linked to esophageal squamous cell carcinoma, endometrial cancer, and glioblastoma [11–13]. While the clinical impacts of RIT1 are evident, understanding of its function and pathological mechanisms at the molecular level remain understudied.

Here, we review current knowledge of the biochemical properties and functional signaling relating to the RIT1 GTPase and provide a phenotypic overview of RIT1 in disease. By discussing the recent efforts that have characterized RIT1 at the molecular, cellular, and organismal level, we hope to establish the need for gaining mechanistic insight into RIT1-mediated pathogenesis.

## Structural and biochemical characterization of RIT1

The crystal structure of GDP-bound RIT1 reveals a globular tertiary fold composed of five alpha helices surrounding a central six-stranded beta-sheet with a fold identical with the G-domain of Ras GTPase family proteins. In addition to its G-domain, RIT1 contains N-terminal and C-terminal extensions; the latter is present in all Ras GTPases and is termed the hypervariable region (HVR) due its low sequence homology. The RIT1 G-domain (residues 19–185) shares a high degree of sequence identity (~51%) with the closely related Ras isoforms HRAS, NRAS, and KRAS (Figure 2A). Like all Ras family GTPases, the RIT1 G-domain contains a set of highly conserved G box guanine nucleotide-binding motif elements (G1–G5) [14]. Based on homology to other Ras GTPases, binding to GTP activates RIT1 by inducing a conformational change in the flexible effector binding interface consisting of switch-I (residues 48–58) and switch-II (residues 78–93). The intrinsic GTPase activity of RIT1 mediates the hydrolysis of bound GTP and the release of γ-phosphate returns switch I and switch II to their inactive ground-state conformation (Figure 2B,C) [15]. Nucleotide exchange occurs through spontaneous release of the bound nucleotide, allowing a new molecule of GDP or GTP to rebind. The slow intrinsic GDP exchange rate ($7.8 \pm 0.7 \times 10^{-2}$ $min^{-1}$), albeit approximately four-fold faster than HRAS, suggests that RIT1 exchange may be under the control of GEFs *in vivo* [16]. Similarly, the slow intrinsic hydrolysis rate ($8.8 \pm 1.3 \times 10^{-3}$ $min^{-1}$) may be regulated by GAPs. However, GEF and GAP enzymes capable of modulating the RIT1 GTPase cycle have not been identified.

RIT1 alleles harboring typical hotspot activating mutations in codons corresponding to G12, G13 and Q61 of HRAS, NRAS, and KRAS (G30, G31 and Q79 in RIT1) are rare in human disease, with a single occurrence of a pathogenic RIT G31R allele driving Noonan syndrome in a familial mother-daughter case [17]. The consequence of a Glycine to Arginine substitution at position 31 on the intrinsic GTPase activity of RIT1 has not been evaluated; however, expression of wild-type and G31R mutant RIT1 cDNA in cells results in comparable MAPK pathway activation [18]. Similarly, the engineered G30V mutation exhibits minimally enhanced activation of the MAPK pathway [7]. The absence of naturally occurring RIT1 Q79 mutations may be explained by the location of Q79 codon at an exon-intron boundary, as previously noted [6]. However, the engineered Q79L mutant has been frequently employed as a constitutively active form of RIT1 in ectopic expression systems due to its impaired GTPase activity *in vitro* and predicted insensitivity to GAP proteins [15]. In support of this observation, several groups have reported the transformation potential of this engineered mutant in classical NIH3T3 transformation assays [6,19,20]. Interestingly, despite its reduced intrinsic hydrolysis rate and elevated exchange rate *in vitro*, when expressed in HEK293T cells, RIT1 Q79L exhibits similar basal levels of GTP loading compared with wild-type in serum-starved cells [21]. The discrepancy between the expected behavior of RIT1 mutations corresponding to oncogenic activating Ras alleles and their observed behavior in cells requires further exploration and suggests that regulation of the RIT1 GTPase cycle *in vivo* may include non-canonical mechanisms.

The predicted effector binding domain of RIT1 is nearly identical with the effector binding domain of Ras but highlights key differences which may explain the differential selectivity for effector and regulatory proteins exhibited by RIT1. A notable difference is the

phenylalanine residue at position 82 of RIT1. The corresponding position on HRAS contains a tyrosine residue that hydrogen-bonds with Lysine 234 of the well characterized Ras effector phosphoinositide 3-kinase gamma (PI3Kγ) [22]. Similarly, Q25 of HRAS, which makes additional polar contacts with the PI3Kγ RAS-binding domain (RBD), is replaced by a serine residue (S43) within the RIT1 sequence (Figure 2D). Although speculative, these differences are likely to compromise RIT1-PI3K binding and may explain why neither of the four Class I PI3K isoforms are direct RIT1 effector proteins [23].

Pathogenic RIT1 mutations decorate the RIT1 G-domain but tend to cluster in or near switch II. Biochemical characterization of a subset of NS missense mutations (S35T, A57G, F82V, T83P, F89H) suggests that these mutations accelerate the RIT1 GTPase cycle, with mutations F82V and T83P primarily decreasing the intrinsic hydrolysis rate, and mutations S35T, A57G, and Y89H primarily increasing the intrinsic nucleotide exchange rate [16]. Importantly, GTP-loading of NS mutants observed *in vitro* positively correlates, albeit weakly, with GTP-loading in cells (Table 1). Although the number of disease-associated mutants evaluated in the study by Fang et al. is limited, it provides important insight into the behavior of mutant RIT1 *in vivo* in the absence of any GAPs or GEFs. As described below, pathogenic RIT1 mutants evade protein turn-over *in vivo* and exhibit enhanced mitogenic signaling, which may be attributed to both mass action and their compromised intrinsic GTPase cycle [21].

Alternative splicing of the *RIT1* gene produces three RIT1 isoforms (Figure 2A). Isoform 2 (NM_006912.6 and NP_008843.1), the canonical isoform used for residue numbering, is a 25.1 kDa protein (219 aa) with an 18-residue extension at the N-terminal end of the G-domain (residues 1–18). Isoform 1 (NM_001256821.2 and NP_001243750.1) contains 17 additional amino acid residues at the N-terminus and exhibits a molecular mass of 27.1 kDa. The function and biochemical properties of the RIT1 N-terminal extensions have not yet been evaluated but could result in differential interacting partners. Exon skipping in transcript variant 3 (NM_001256820.2 and NP_001243749.1) produces a 21.6 kDa protein with a translation initiation corresponding to M37 of isoform 1. The partial G-domain of isoform 3 lacks the G1 box sequence, also known as a P-loop, and is predicted to exhibit impaired nucleotide binding and compromised protein stability.

A distinctive feature of the RIT1 HVR is the lack of a CAAX box motif or Cysteine residue(s) that would allow anchoring of RIT1 to the plasma membrane via lipid moieties. Despite this, the RIT1 HVR is sufficient and necessary for plasma membrane association due to the presence of positively charged amino acid clusters that mediate electrostatic interactions with negatively charged membrane phospholipids [24,25]. While it is the polybasic nature of the RIT1 HVR that provides the energy required for plasma membrane association, hydrophobic residues flanking each polybasic cluster are responsible for selective targeting of RIT1 to the plasma membrane [25]. The lack of a prenylation motif suggests that the RIT1-plasma membrane association may be transient in nature and highly sensitive to local changes in negative charge of membrane lipids. Indeed, Heo et al. demonstrate that simultaneous depletion of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] and phosphatidylinositol 3,4,5-triphosphate [$PI(3,4,5)P_3$] markedly dissociates RIT1 HVR from the plasma membrane. These studies were conducted with chimeric

fluorescent proteins fused to the C-terminal tail of RIT1 and it remains unclear whether the G-domain of RIT1 provides additional binding energy to the plasma membrane via direct interaction with membrane lipids or through its interaction with membrane-interacting effector proteins, such as RAF [2]. Our current understanding of the behavior of RIT1 at the cytoplasmic face of the plasma membrane is limited. Nanoclustering of Ras proteins is heavily influenced by the biophysical properties of their HVR [26]. How the unique properties of the RIT1 HVR influence its lipid microenvironment and whether its selectivity for lipids overlaps with other Ras GTPases, remains to be determined.

## Regulation of cellular function and downstream signaling by RIT1

Comparison of sequence identity, especially in the effector domain, between RIT1 and Ras suggest overlapping and unique interactors responsible for the signaling properties of each GTPase. The earliest efforts to identify these properties, however, contrast with what is known about RIT1 signaling today, perhaps contributing to the existing obscurity of RIT1 function. For example, a yeast two-hybrid system was used to screen for effectors of RIT1 using a library of known Ras effectors, including RAF kinases, the RBDs of RLF and RalGDS, AF6, and the p110 catalytic subunit of phosphatidylinositol 3-kinase (PI3K). Surprisingly, no interactions were detected between RIT1 and the RAF paralogs nor with PI3K; moreover, it was believed soon after that RIT1 did not activate ERK, JNK, p38 MAPK or PI3K/AKT pathways as shown by a transient immune complex kinase assay in COS monkey cells [15,20]. However, in a separate study, rat pheochromocytoma (PC6) cells infected with adenovirus encoding the RIT1 Q79L constitutively active mutant exhibited increased phosphorylation of ERK via immunoblotting [27]. In another study, GST pulldown from COS cells showed that RIT1 does interact with both BRAF and CRAF (also known as RAF1) [28]. The contrasting conclusions between studies are a recurring theme, which represent the early challenges of characterizing RIT1 function, and can be attributed to the sensitivity of techniques as well as the difference in cellular systems. Later studies have provided further evidence suggesting that RIT1 appears to signal indirectly through p38 MAPK, AKT, and ERK, as well as other unique pathways that control key cellular processes (Figure 3). Notably, many of these experiments are based on RIT1 overexpression and mutations that do not occur in nature, such as the Q79L and S35N missense mutations that are analogous to constitutively active and dominant-negative forms of Ras, respectively. Since the effects of wild-type RIT1 are difficult to characterize and are seldom investigated, ‘artificial’ and pathogenic mutations may therefore reflect neomorphic RIT1 activity.

The earliest identified functions of RIT1 are neuronal growth and differentiation via signaling through both p38 MAPK and RAF/MEK/ERK pathways. The p38 MAPK cascade is a major signaling pathway activated by stress and mitogenic stimuli and has been connected to cellular responses such as cytokine production, apoptosis, and cell survival [29]. While investigating the transforming activity of RIT1 in NIH3T3 cells, Sakabe et al. provided the first evidence of p38 activation by constitutively active RIT1 via an *in vitro* kinase assay. Moreover, p38γ, but not other p38 isoforms (α, β, δ), was selectively activated and RIT1-induced transformation was subsequently attenuated with the ectopic expression of dominant-negative MKK3, which is upstream of p38 [19]. This suggests that the activation of p38γ by RIT1 occurs either at the MKK3 level or upstream of it. On the other

hand, a later study found that RIT1 Q79L activated both p38α and p38γ in PC6 cells [30]. Although the selective activation of p38 isoforms have not been further examined, especially in human cells, other studies continued to expand on RIT1-mediated p38 activation in the context of neuronal behavior, as well as cellular stress responses.

Neuronal signaling by RIT1 has been characterized in more detail using cellular systems such as PC6 cells, hippocampal neurons, and sympathetic neurons. Spencer et al. first demonstrated that RIT1 Q79L potentiated MEK-dependent neurite outgrowth in PC6 cells and confirmed that RIT1 acted upstream, or at the level of RAF, using dominant-negative mutants of RIT1 and MEK1 [27]. Further analysis in PC6 cells demonstrated that RIT1, which can be activated by NGF and EGF in these cells, preferentially activated BRAF, but not RAF1, as well as p38 MAPK to induce neuronal differentiation [28]. Similar findings have been implicated under the context of pituitary adenylate cyclase-activating polypeptide (PACAP38)-induced signaling in PC6 cells, which is known to stimulate neuronal differentiation and outgrowth. Silencing of wild-type RIT1 by shRNA reduced PACAP38-mediated activation of p38, but not ERK, and attenuated neurite branching and growth. Furthermore, EPAC1, which act as cAMP-dependent GEFs for Rap GTPases, played an indirect role in PACAP38-mediated RIT1 activation, since EPAC1 silencing led to lower levels of GTP-bound RIT1 [30]. A later report suggested that in this context, EPAC1 leads to Src-dependent TrkA transactivation and subsequent SOS1/2-mediated RIT1 activation. Like EPAC1, SOS1/2 failed to act as a direct RIT1 GEF *in vitro* [31].

To extend analysis from PC6 cells, which are derived from rat, to a more physiologically relevant context, Hynds et al. used the human neuroblastoma cell line SH-SY5Y to demonstrate that RIT1 exhibited MEK-independent and MEK-dependent effects on neurite branching and initiation, respectively. Specifically, MEK inhibition using the chemical inhibitor PD098059 or expression of a MEK1 dominant-negative mutant in RIT1 Q79L-expressing cells reduced the number of neurites in each cell and the percent of neurite-bearing cells, but not the total neurite length or number of branch points in each neurite [32]. Further quantification in RIT1 Q79L-expressing PC6 cells suggest that the MEK-independent effects of neurite branching are mediated by p38, since inhibition with the p38 inhibitor SB203580 resulted in a reduction in neurite length and branching, but not neurite initiation [28].

Consistent with the notion that RIT1 regulates both p38 and MEK/ERK signaling to control different aspects of neurite outgrowth, studies using hippocampal and sympathetic neurons have demonstrated that RIT1 also differentially controls axonal and dendritic growth. For example, sympathetic neurons expressing constitutively active RIT1 enhanced axonal growth, but inhibited NGF- and BMP7-induced dendritic growth. The axon-promoting and dendritic-inhibiting effects of RIT1 Q79L were blocked by a MEK inhibitor, supporting the involvement of MEK/ERK signaling in this process. Moreover, Lein et al. speculated that BMP7 may induce dendritic growth by increasing the ratio of inactive to active state of RIT1. GST pulldown assays precipitating active RIT1 from PC6 cells via RGL3-RBD showed that BMP7 decreased GTP-bound RIT1 levels but only when those cells were pretreated with NGF. Otherwise, BMP7 alone increased RIT1-GTP levels [33]. However, this treatment paradigm may not be physiologically relevant and the *in vivo* regulation of

nucleotide-bound RIT1 remains to be characterized. Whereas BMPs induce dendritic growth in sympathetic neurons, IFNγ inhibits it and triggers dendritic retraction, much like the phenotypic effect seen with RIT1 Q79L expression. In both hippocampal and sympathetic neurons, the expression of dominant-negative RIT1 has been shown to block IFNγ-induced dendritic retraction. IFNγ induced RIT1 activation, as indicated by increased levels of co-precipitated RIT1 from GST-RGL3-RBD, in both PC6 and hippocampal neurons, yet IFNγ failed to stimulate ERK activation. Instead, IFNγ induced p38 activation through RIT1 and when treated with SB203580, dendritic retraction was attenuated, revealing an IFNγ-RIT1-p38 signaling pathway [34].

In addition to p38 MAPK and MEK/ERK activation, RIT1 has also been found to promote AKT activation to regulate hippocampal neurogenesis. In mice, RIT1 Q79L overexpression resulted in stabilization of Sox2 protein, a transcription factor highly involved in adult neurogenesis. Meanwhile, pharmacological inhibition of AKT revealed that RIT1-mediated Sox2 activation relied on AKT signaling [35,36]. The scaffolding complexes and molecular mechanisms that control pathway-specific RIT1 signaling within and between cell types remain unknown.

In addition to modulating neurotrophic signaling and neuronal morphogenesis, RIT1 function has been implicated in p38-mediated stress response and survival in PC6 cells, mouse embryonic fibroblasts (MEFs), and hippocampal neurons. RIT1-targeted shRNA enhanced apoptosis in PC6 cells in response to a variety of cellular stresses such as actinomycin D, hydrogen peroxide, TNFα, etoposide, and tunicamycin [37]. MEFs isolated from RIT1 null mice, however, were less viable under reactive oxygen species (ROS) exposure, but not to etoposide or tunicamycin treatment [38]. Importantly, p38 and MAPKAPK2 (MK2) complex together to allow MK2 to phosphorylate HSP27 and AKT [39–41]. In PC6 cells and MEFs deficient for RIT1, phosphorylation of MK2, HSP27, and AKT was reduced following hydrogen peroxide exposure. Moreover, pharmacological blockade of p38 by SB203580 phenocopies these effects in WT MEFs and in PC6 cells expressing constitutively active RIT1 [37,38]. Further characterization of RIT1 involvement in p38-mediated ROS-dependent AKT activation found that mTORC2, but not mTORC1 is essential for this cascade [42]. The preservation of RIT1-mediated oxidative stress response in hippocampal neurons was then investigated in another study, which found that neural cultures from newborn RIT1 null mice displayed reduced ROS-dependent p38 and AKT activation, as well as impaired neurogenesis following controlled cortical injury in the dentate gyrus of the ipsilateral hemisphere. However, loss of RIT1 did not cause apparent morphological or developmental changes to the hippocampus [43]. These studies outline consistent RIT1 signaling in multiple cell types and show a conserved role for RIT1 in mediating p38-AKT oxidative stress response in flies and mice.

In contrast with Ras, RIT1 has been described to interact with unique effectors that regulate actin dynamics, which may have pathophysiological relevance in NS or cancer. Par6 is a regulator of cell polarity that complexes with GTP-bound Cdc42 and Rac1 through a semi-Cdc42/Rac interactive binding (CRIB) domain. Initial findings demonstrated in COS7 cells that RIT1 forms a ternary complex with Cdc42/Rac through a direct GTP-dependent interaction with the PDZ domain of Par6 [44]. While the study did not distinguish between

Par6 isoforms, it was later confirmed that RIT1 interacts with Par6 isoforms A, B, and C. However, downstream analyses which focused on the Par6C isoform revealed that RIT1 S35N, which is unable to load GTP, as well as recombinant RIT1 loaded with either GDP or GTPγS, potently bound to Par6C. Moreover, association of RIT1 S35N with Par6C did not require an intact PDZ domain [45]. The contradicting dependence of RIT1 activation on Par6 interaction is not understood and may suggest that other cellular factors contribute to the complexity of this scaffolding complex. Recently, *in vitro* binding assays have shown that RIT1 also interacts directly with Cdc42, Rac1, and PAK1 in a nucleotide-independent manner. Like Par6, PAK1 has a CRIB domain and acts downstream of Rho family members. Whereas the previously described studies used 'artificial' mutations of RIT1 (Q79L, S35N), others have sought to understand the effect of NS-associated RIT1 mutations on actin dynamics. Co-immunoprecipitation assays from HEK293T and COS7 cells showed that these RIT1 mutants have increased binding to the CRIB domain of PAK1. Additionally, the NS-associated mutants displayed enhanced binding under serum-deprived (0.1%) but not basal (10%) serum conditions. Immunocytochemistry in COS7 cells reveals that ectopic expression of WT or mutant RIT1 caused stress fiber dissolution and disassembly of focal adhesions, which can be prevented by co-expression of dominant-negative PAK1 K299A, Cdc42 S17N, or Rac1 S17N. Despite the similar actin dynamic effects between overexpressed WT RIT1 and NS-associated mutants, the study nevertheless provided further evidence supporting the role of RIT1 in cyto-skeletal rearrangement [18]. Future studies in mice with endogenous expression of mutant RIT1 may distinguish if disease-associated RIT1 alleles confer gain-of-function effects in this context.

Recent work in our lab has identified LZTR1 as a unique RIT1 interactor, providing RIT1 negative regulation at the protein level through K48-mediated ubiquitination and proteasomal degradation [21]. LZTR1 is a Kelch and BTB-BACK domain-containing protein that acts as a substrate-specific adaptor for the cullin 3 RING E3 ubiquitin ligase (CUL3) and like RIT1, is often mutated in Noonan syndrome and cancer. When compared with other Ras family members, LZTR1 interaction was only preserved with MRAS. Although RIT1 WT and Q79L displayed GDP-dependent interaction with LZTR1, RIT1 oncoproteins primarily mutated within the switch II region exhibited no detectable LZTR1 interaction, suggesting that this region could represent the interacting interface. Consistent with this, RIT1 ubiquitination and degradation by LZTR1 was decreased in the pathogenic mutants, and LZTR1 knockout MEFs exhibited increased amounts of RIT1 protein levels compared with WT and heterozygous littermates [21]. In a separate study, cells expressing RIT1 mutants were shown to display increased half-lives when compared with WT, which could now be explained in part by impaired LZTR1 activity on RIT1 mutants [46]. In line with these findings, Wang et al. independently provided equivalent evidence of RIT1-specific proteolysis by LZTR1 and extended the characterization to glioblastoma cells. Deletion of LZTR1 in glioblastoma cells resulted in enhanced MEK/ERK signaling, which was also observed in LZTR1 null MEFs [21,47], suggesting that LZTR1 negatively regulates MEK/ERK activation through RIT1.

## RIT1 in animal models

Human RIT1 orthologs are found in many model organisms, such as Drosophila, zebrafish, and the mouse. Therefore, these species have been instrumental to elucidate the roles of RIT1 GTPase at the organismal level. In the fruit fly *Drosophila melanogaster*, where the ortholog RIC was isolated from a cDNA library derived from the fly retina [5], several models have been described. For instance, RIC null flies were generated by imprecise excision of the transposon P{RS5}5-HA-1205, which is adjacent to the RIC coding sequence. Different lines containing a RIC deletion have been characterized and found to be fertile and normal during development and embryogenesis, with no appreciable phenotypes [38]. However, RIC null flies are more susceptible to environmental stress, exhibiting decreased survival to osmotic stress, heat shock, dry starvation, and oxidative stress. In a different study, expression in Drosophila of the RIC mutation Q117L, which is equivalent to the constitutively active RAS mutation Q61L, led to Ras-related phenotypes. When $RIC^{Q117L}$ is expressed in the wing or the eye using the GAL4/UAS binary system, flies develop wing ectopic veins and roughened eyes, respectively [48]. Using this convenient wing model, several alleles have been tested for the ability to rescue or exacerbate the ectopic vein phenotype. Consistent with previous observations suggesting that this phenotype is largely dependent on MAPK constitutive activation [49,50], loss-of-function alleles in the Ras ($Ras85D^{eIB}$), MEK ($MEK^{LH110}$), and SOS ($Sos^{X122}$) orthologues partially rescued the phenotype [48]. Interestingly, hypomorphic alleles of cam, the calmodulin fly ortholog, led to an increased phenotype in this system, suggesting that this protein acts as a negative regulator of RIC-dependent MAPK activation.

In the zebrafish *Danio rerio*, injection of antisense RNA against RIT1 in the one-cell stage embryo did not yield any phenotype during gastrulation or in older embryos (52 hpf). However, this model was valuable to assess the effect of the human RIT1 pathogenic variants. Injection of mRNA encoding for the human pathogenic variants E81G, G95A, M90I, and A57G, as well as the constitutively active Q79L, has been shown to result in gastrulation defects, facial and head abnormalities, and heart anomalies (including incomplete looping, hypoplastic chambers, and stagnation of blood flow in the yolk sac) [7,51]. To date, no zebrafish models have been reported carrying RIT1 knockout or knock-in alleles in the germline. In contrast, a mouse model carrying a loss-of-function allele has been previously described; this strain, which contains a LacZ cassette replacing the coding RIT1 exon 2, results in a complete knockout when in homozygosity [38]. RIT1 knockout mice are born at the expected Mendelian ratios and do not show morphological or histological abnormalities. This suggests that RIT1 is not required for the normal development of the mouse. However, embryonic fibroblasts derived from these mice exhibit increased sensitivity to oxidative stress, in line with the observations made in RIC null flies [38]. The lack of phenotype in RIT1 knockout mice and flies and the increased sensitivity of these mutants in response to cellular stress suggests that, in organisms, RIT1 might function as a key molecular factor to overcome these negative cues. Therefore, it would be interesting to test whether RIT1 knockout mice are more susceptible to certain conditions associated with increased cellular stress, such as ischemia/reperfusion, traumatic injury, neurodegeneration, or ageing [52,53].

Several mouse models have been engineered to carry RIT1 gain-of-function mutations. A transgenic strain containing the RIT1$^{Q79L}$ variant under the control of the tetracycline operator has been described in the literature. This mouse line, when crossed to other strains carrying a tetracycline-controlled transactivator (tTA) under the control of a specific promoter, allow the overexpression of RIT1 Q79L in a doxycycline-inducible manner. This is exemplified by a model in which RIT1 is overexpressed in the central nervous system by using a tTA regulated by the $Ca^{2+}$-calmodulin-dependent kinase II promoter [35]. Although no morphological phenotype has been described for these animals, the adult hippocampal neural progenitor cells of these transgenic mice exhibit increased proliferation and Sox2-dependent transcriptional activity.

Our laboratory has developed a conditional knock-in mouse strain that contains the RIT1 M90I mutation in the endogenous locus. In this model, a minigene containing exons 4–6 were flanked by loxP sites, while the downstream endogenous exon 5 was mutated to contain the M90I variant. When crossed to mice carrying the Cre recombinase, the wild-type minigene is excised allowing the expression of the mutant version [21]. Mice crossed with the CMV-Cre strain, resulting in germline expression of RIT1 M90I, exhibit a phenotype that resembles NS. In this characteristic phenotype, mice show craniofacial dysmorphia (e.g. blunt snout, hypertelorism, and rounded skull), decreased body weight and length, and enlarged heart and spleen. While the enlarged heart seems to be the result of hypertrophic cardiomyocytes, splenomegaly is caused by extramedullary hematopoiesis. These findings are consistent with other mouse models of NS that have been previously generated, such as the ones expressing the germline variants SHP2 D61G, RAF1 L613V, and KRAS V14I [54–56].

Another NS pathogenic mutation of RIT1 has been used to generate an alternative mouse model of this developmental disorder. The A57G variant, which is frequent in NS but not in cancer, leads to a similar phenotype when expressed in the germline. Interestingly, these mice presented with cardiac fibrosis and an increased number of activated cardiac fibroblasts and myofibroblasts [57]. Moreover, RIT1 A57G mice exhibited increased cardiac fibrosis upon treatment with the non-selective β-adrenergic receptor agonist isoproterenol, which has been shown to cause myocardial injury in mice [58]. Both the RIT1 M90I and RIT1 A57G models have reported a severe cardiac phenotype, which is consistent with the phenotype observed in patients. The cardiac involvement appears to be mediated by RAF/MEK activation, because patients and mice with activating RAF1 mutations present with similar hypertrophic findings [55,59] and treatment of RIT1 mutant NS patients with MEK inhibitors appear to be beneficial in this setting [60].

Genetically engineered mouse models in which the role of RIT1 mutations have been studied as cancer drivers have not been published yet. However, there are two mouse models that take advantage of cell lines carrying such mutations engrafted in immunocompromised mice. The first using a xenograft of the lung adenocarcinoma cell line NCI-H2110, which contains the M90I variant, and the second by allografting NIH3T3 mouse fibroblasts transformed with pathogenic RIT1 variants [6]. While these models can have limited application for understanding the biology underlying RIT1-mediated transformation, they can be valuable platforms to identify therapies aimed at targeting RIT1 mutant tumors.

## RIT1 in disease

RIT1 mutations have been identified in a number of different human conditions, including cancer and NS. The latter belongs to a large group of disorders termed RASopathies, which are syndromes characterized by germline mutations in the RAS/MAPK pathway [4]. In the past decade, RIT1 has emerged as a causative gene in NS with mutations accounting for at least 5% of genetically confirmed cases [17], exceeded only by PTPN11 (~50%) and SOS1 (~10%) and comparable to RAF1 (~5%) [61]. So far, the most common RIT1 missense mutations in NS comprise of A57G, F82L, and G95A (Figure 4A,B) [7,17,46,51,60,62–75], suggesting specialized molecular functions around these residues. Biochemical characterizations, for example, reveal that these residues affect the GTPase cycle of RIT1. Specifically, A57G and F82V confer increased intrinsic nucleotide exchange and reduced GTP hydrolysis, respectively. In contrast, G95A displays impaired stability *in vitro* and reduced expression *in vivo* likely caused by restriction of the switch II region and defective nucleotide binding [16]. On the other hand, somatic mutations which partly overlap with NS-associated mutations have also been recently identified in cancer. Altogether, the majority of pathogenic missense mutations in RIT1 cluster at or near the switch II region.

NS is typically characterized by a broad spectrum of clinical manifestations including craniofacial dysmorphia, short stature, congenital heart defects, hematological and lymphatic anomalies, and intellectual disability [61]. The genotype–phenotype relationships in RIT1-mutated NS patients has been explored by multiple groups, despite small cohort sizes. For instance, while craniofacial features are typical of NS, short stature, ptosis, pectus excavatum, and ectodermal abnormalities have been observed less frequently in RIT1-mutant patients. Additionally, these patients seem to have milder learning disabilities relative to other NS cohorts. On the other hand, high birth weight, perinatal lymphatic abnormalities including nuchal translucency, polyhydramnios, lymphedema, and fetal hydrops, and cardiovascular defects are highly consistent features of RIT1 mutant patients [7,17,46,62,65,70,71,76]. Pulmonic stenosis, atrial septal defect, and hypertrophic cardiomyopathy (HCM) are among the most common cardiac abnormalities in NS patients and are the primary contributors to mortality and morbidity [77]. Approximately 20% of NS patients have HCM and have a significantly higher mortality rate than non-syndromic children with HCM [61,77,78]. Importantly, HCM is much more prevalent in RIT1-mutant NS patients with an incidence of 54% and is only exceeded by RAF1-mutant patients (~75%) as the most frequent gene associated with HCM in NS [71]. This may suggest that RIT1 and RAF1 co-ordinate similar signaling processes contributing to cardiovascular development. Indeed, we reported interaction between RIT1 disease-associated mutants and RAF1, although this interaction was weak [21]. Recently, off-label MEK inhibition using the FDA-approved trametinib was used to treat two NS patients containing RIT1 mutations, S35T and F82L, with severe, early-onset HCM. HCM regression was observed in both patients, thus supporting pathogenic MEK activation by RIT1 [60]. Moreover, a recent study has described the generation of RAF1-mutant inducible pluripotent stem cell-derived cardiomyocytes that phenocopy HCM in NS. In this system, hyperactivation of MEK1/2, but not ERK1/2 affected cardiomyocyte structure, and ERK5 signaling contributed to enlargement of the cardiomyocyte [79]. Whether RIT1 mediates ERK5 activation in

cardiomyocytes is currently unknown. Moreover, the molecular mechanisms underlying HCM are likely to be complex and not only cardiomyocyte specific, since it has been shown that interplay with endothelial cells is necessary for cardiomyocyte hypertrophy [80]. Taken together, many groups have recognized the high prevalence and severity of congenital heart defects in NS patients carrying RIT1 gain-of-function mutations. However, the biochemical mechanism of MAPK activation by RIT1 is still unclear and should be elucidated especially in the context of HCM.

NS patients are prone to developing certain types of cancer, especially during childhood, and can present with hematopoietic disorders [81]. Indeed, germline RIT1 mutations in some of these patients have been associated with acute lymphoblastic leukemia (ALL) and juvenile myelomonocytic leukemia [7,63]. While there is no current evidence suggesting that these patients are more susceptible to developing leukemia than other NS patients [63], somatic mutations and locus amplifications in RIT1 have been identified in patients with myeloid neoplasms such as chronic myelomonocytic leukemia [82]. RIT1 variants have also been described in other cancers including lung adenocarcinoma, salivary gland carcinoma, and endometrial carcinoma. Expression analyses have shed light onto the pathogenic role of RIT1 in various cancers. In hepatocellular carcinoma (HCC), RIT1 amplification is found in 25% of affected patients, and up-regulation of RIT1 correlated with poorer prognosis as indicated by Kaplan–Meier survival analysis [83,84]. RIT1 promoted HCC cell proliferation and metastasis *in vitro* and *in vivo*, and its deficiency could confer sensitivity to sorafenib treatment in cell lines [84]. Likewise, RIT1 mRNA and protein levels were significantly elevated in endometrial cancer (EC) cell lines and tissue samples, and high RIT1 expression was associated with poor overall survival [12]. Analysis from datasets reveal that RIT1 is most significantly up-regulated in glioblastomas relative to normal brain tissues and is correlated with poor survival especially in low grade glioma [13]. However, unlike HCC, EC, and glioma, RIT1 appears to be down-regulated in esophageal squamous cell carcinoma (ESCC), and low expression was significantly associated with poorer prognosis. In ESCC, RIT1 overexpression promoted inhibition of epithelial-mesenchymal transition and increased drug sensitivity to cisplatin [11]. The distinct expression and mutational profile of RIT1 exists in diverse cancers and is not strictly limited to specific tissues. While RIT1 has been shown to have a potential prognostic value, the molecular and pathogenic mechanisms by which RIT1 acts in specific biological contexts remains understudied.

## Conclusions and future directions

RIT1 has emerged as an important GTPase in human pathogenesis because gain-of-function mutations in its gene are associated with cancer and the developmental disorder, NS. To date, most experimental evidence suggests that RIT1 is a GTPase that regulates neuronal growth and differentiation, as well as stress response in different cell types. Although there is an increasing number of studies that have identified downstream effectors of RIT1, there is a lack of consensus regarding the major signaling outputs of activated RIT1. Similarly, the physiological mechanisms that promote nucleotide exchange and hydrolysis, as well as the GEF and GAP enzymes that catalyze these processes, remain unknown. Given the relationship of NS with the RAF/MEK/ERK pathway, it is fair to accept that RIT1 is a regulator of this pathway and activating mutants contribute to its dysregulation. Since the

organismal models that lack this protein do not exhibit striking phenotypes, but those with activating mutations do, it can be speculated that the functions of RIT1 in the physiological setting are transient and acute; perhaps, by promoting the activation of multiple pro-survival pathways in response to environmental or cellular stress. Therefore, future work will be required to address the fundamental questions that address how RIT1 is regulated at the molecular level, by which factors, and in which settings.

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## Abbreviations

| | |
|---|---|
| **CRIB** | Cdc42/Rac interactive binding |
| **EC** | endometrial cancer |
| **ESCC** | esophageal squamous cell carcinoma |
| **GAP** | GTPase-activating protein |
| **GDP** | guanosine diphosphate |
| **GEF** | guanine nucleotide exchange factor |
| **GTP** | guanosine triphosphate |
| **GTPase** | guanosine triphosphate hydrolase |
| **HCC** | hepatocellular carcinoma |
| **HCM** | hypertrophic cardiomyopathy |
| **HVR** | hypervariable region |
| **MAPK** | mitogen activated protein kinase |
| **MEF** | mouse embryonic fibroblast |
| **MK2** | MAPKAPK2 |
| **NS** | Noonan syndrome |
| **PACAP38** | pituitary adenylate cyclase-activating polypeptide |
| **PC6** | rat pheochromocytoma |
| **PI3K** | phosphoinositide 3-kinase |
| **RBD** | RAS-binding domain |
| **ROS** | reactive oxygen species |

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| **tTA** | tetracycline-controlled transactivator |

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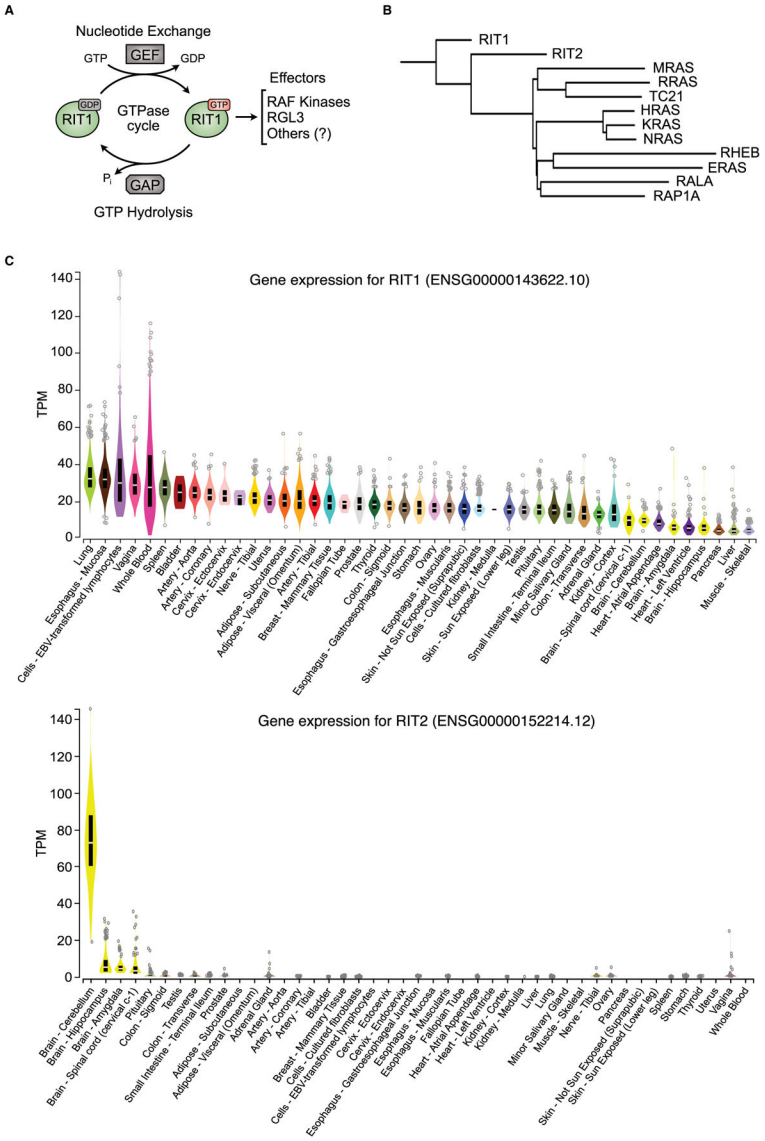


**Figure 1. Overview of RIT1 GTPase.**

(**A**) The nucleotide cycle of RIT1 is similar to most Ras GTPases. Upon nucleotide exchange promoted by unknown GEF, GTP-loaded RIT1 interacts with specific effectors. Hydrolysis of bound GTP is mediated by the intrinsic GTPase activity and catalyzed by unknown GAP. (**B**) Dendrogram showing relationship between the protein sequence of RIT1 and other GTPases. Uniprot protein sequences were aligned using Clustal Omega and dendrogram was generated with iTOL [85,86]. (**C**) Expression of *RIT1* and *RIT2* mRNA in different human tissues of the GTEx database (dbGaP Accession phs000424.v8.p2) [9]. TPM, transcript per million reads.

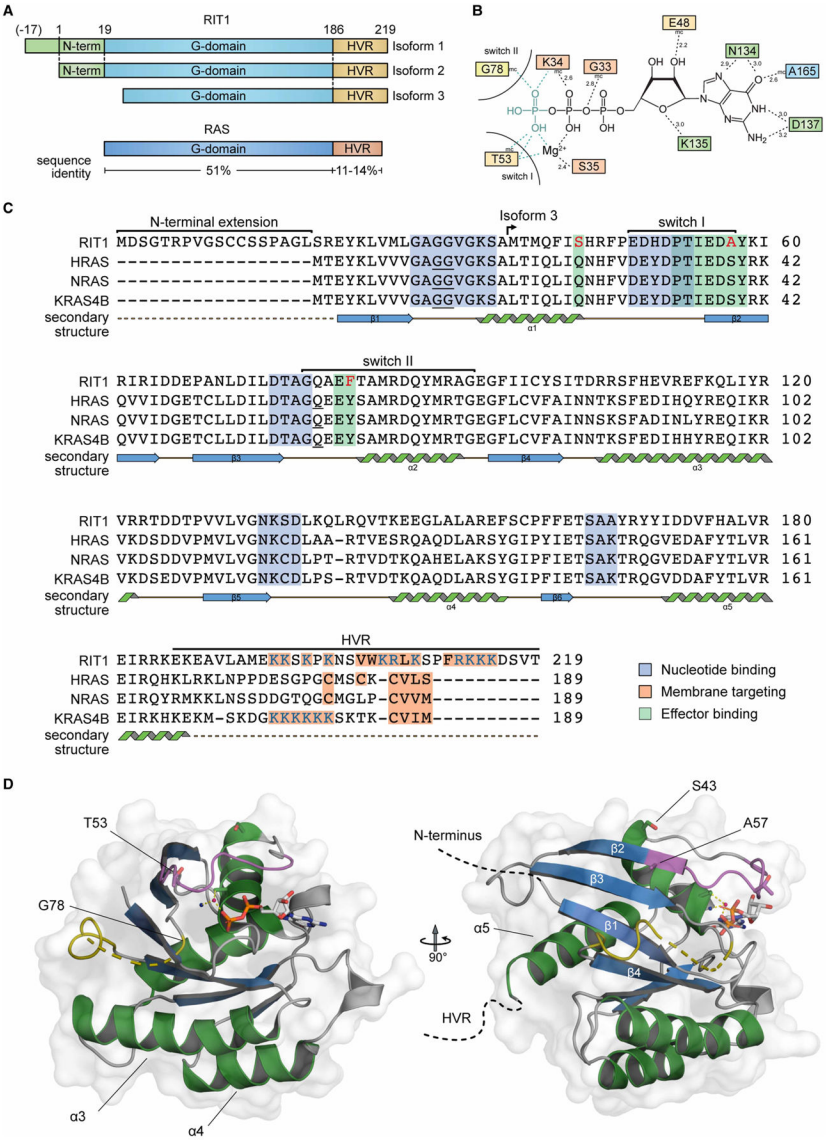


**Figure 2. Biochemical characteristics of RIT1 GTPase.**

(**A**) *RIT1* gene gives rise to three different RIT1 isoforms, which exhibit different N-terminal domains. HVR, hypervariable region. (**B**) RIT1 aa involved in nucleotide binding are depicted. Polar contacts with gamma phosphate (teal) are predicted based on homologous contacts in GTP-bound RAS [87]. (**C**) Alignment between RIT1 (isoform 2), NRAS, HRAS, and KRAS (isoform 4B) aa sequence is shown. Different aa involved in nucleotide (blue), effector (green) binding, and membrane targeting (orange) are highlighted. (**D**) Overall view of RIT1 crystal structure. Switch I is shown in purple; switch II is shown in yellow. (PDB:4KLZ).

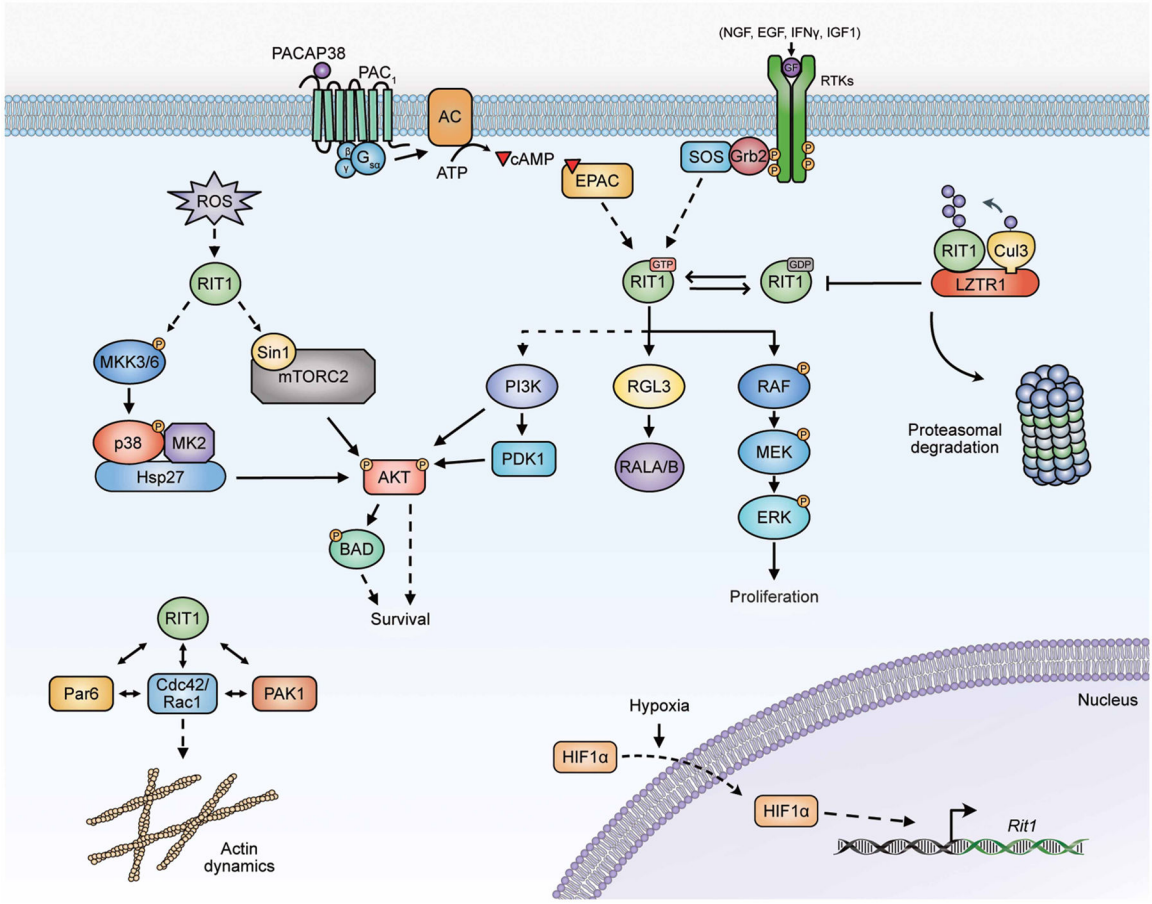


**Figure 3. Signal transduction pathways regulated by RIT1.**

RIT1 is activated in response to certain growth factors, reactive oxygen species, hypoxia, and activating pathogenic mutations. It is negatively regulated by the LZTR1/CUL3 degradation complex and positively regulated by EPAC. GTP-loaded RIT1 activates the RAF/MEK/ERK signaling cascade, the RAL GEF RGL3, PI3K/AKT pathway, and Actin dynamics. The functions of RIT1 are likely to be cell-type dependent but appear to regulate cell survival.

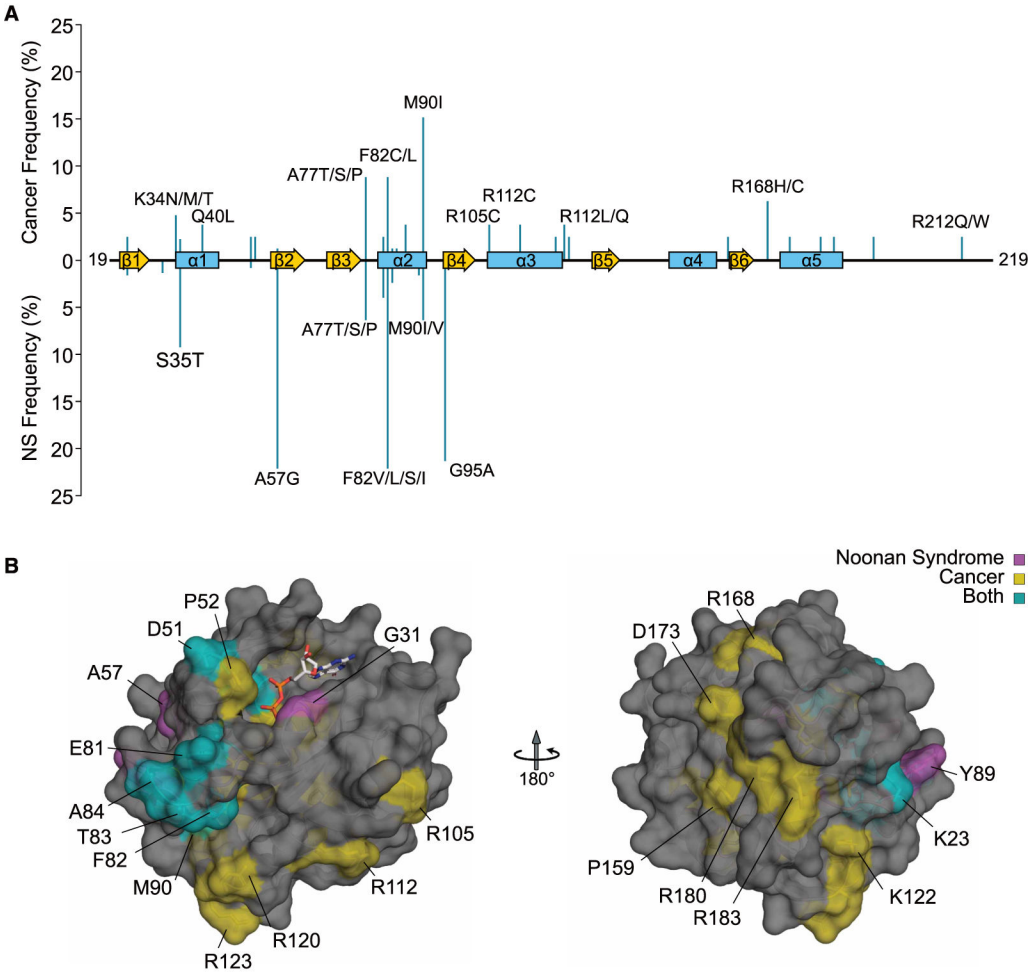


**Figure 4. RIT1 mutations in cancer and NS.**

(**A**) Frequency of disease-associated RIT1 mutations identified in cancer (above) and NS (below). RIT1 single nucleotide variants represented here were obtained from cBioPortal database [88,89] for cancer ($n$ = 79; only alleles found in more than one tumor) and NSEuroNet database web site, www.nseuronet.com for NS ($n$ = 124). (**B**) Schematic representation of allele distribution across the three-dimensional surface of GDP-bound RIT1 (PDB: 4KLZ). The aa that encode for different cancer (yellow), NS (purple), or both (green) mutations are shown.

## Table 1

Biochemical properties of disease-associated RIT1 alleles

| Allele | Exchange[1] ($min^{-1}$) | Hydrolysis[1] ($min^{-1}$) | $k_E/k_H$ | $k_E/k_H$ fold-change | RBD pull down[1] | % GTP *in vivo*[2] |
|---|---|---|---|---|---|---|
| WT | 0.078 | 0.0088 | 8.86 | 1.00 | 1 | 27 |
| S35T | 0.3 | 0.0036 | 83.33 | 9.40 | 0.98 | - |
| A57G | 0.47 | 0.0054 | 87.04 | 9.82 | 4.3 | 40 |
| F82V | 0.076 | 0.003 | 25.33 | 2.86 | 1.2 | 28 |
| T83P | 0.15 | 0.0029 | 51.72 | 5.84 | 2 | 78 |
| Y89H | 0.32 | 0.0057 | 56.14 | 6.33 | 5.6 | 60 |

[1] See Ref [16];

[2] See Ref [21].