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Emerging roles for Regulator of G Protein Signaling 2 in (patho)physiology

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Running title: RGS2 in (patho)physiology

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Abbreviations: RGS, Regulator of G protein signaling; GPCR, G protein-coupled receptor; GAP, GTPase activating protein; AC, adenylate cyclase

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Abstract

Since their discovery in the mid-1990's, Regulator of G protein Signaling (RGS) proteins have emerged as key regulators of signaling through G Protein-Coupled Receptors (GPCRs). Among the over 20 known RGS proteins, RGS2 has received increasing interest as a potential therapeutic drug target with broad clinical implications. RGS2 is a member of the R4 subfamily of RGS proteins and is unique in that it is selective for $G\alpha_q$. Despite only having an RGS domain, responsible for the canonical GTPase Activating Protein (GAP) activity, RGS2 can regulate additional processes, such as protein synthesis and adenylate cyclase activity, through protein-protein interactions. Here we provide an update of the current knowledge of RGS2 function as it relates to molecular mechanisms of regulation as well as its potential role in regulating a number of physiological systems and pathologies, including cardiovascular disease and central nervous system disorders, as well as various forms of cancer.

Significance Statement: RGS proteins represent an exciting class of novel drug targets. RGS2, in particular, could have broad clinical importance. As more details are emerging on the regulation of RGS2 in various physiological systems, the potential utility of this small protein in therapeutic development is increasing.

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Introduction

A vast number of physiological processes are controlled by the large family of G protein-coupled receptors (GPCRs). These receptors mediate signal transduction through the activation of heterotrimeric (αβγ) G proteins. Receptor activation induces a conformational change in Gα that enables the exchange of GDP for GTP, rendering it active and able to mediate downstream signaling cascades. Recently, much effort is being devoted to the mechanisms regulating GPCR signaling. One key regulatory mechanism is mediated by the Regulators of G protein Signaling (RGS) protein superfamily. At least 20 RGS proteins have been identified since the mid-1990s, all containing a conserved 120 residue RGS domain. This domain is responsible for the canonical action of RGS proteins, to serve as GTPase accelerating proteins (GAPs) on active, GTP-bound Gα subunits. Through this action, RGS proteins modulate GPCR signaling by shortening the duration and amplitude of GPCR-mediated responses. In addition, many RGS proteins can regulate cellular functions through non-canonical mechanisms. RGS proteins achieve selectivity towards certain pathways through discrete tissue distribution and selectivity towards Gα subtypes. Thus, they assist in fine-tuning GPCR signal transduction and represent a family of potential new drug targets.

RGS2 is a member of the largest family of RGS proteins, the R4 family. Like all the members of this family, RGS2 is a small protein with no additional domains apart from the RGS domain. Despite this, additional functions other than GAP activity have been attributed to RGS2, such as the participation in protein-protein interactions that affect both GPCR signaling and other processes. Emerging data from *in vitro*, *in vivo* and human studies have identified a wide variety of physiological roles for RGS2 (**Figure 1**). Notably, low RGS2 protein levels have been observed and associated with a variety of disease states. In some cases, the mechanism by which

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RGS2 would contribute to disease has been established; however, much is yet to be discovered about the molecular mechanisms regulating RGS2 and how RGS2, in turn, affects physiological function. Here, we summarize the key features pertaining to cellular functions regulated by RGS2, the regulation of RGS2 protein expression and activity, its physiological roles, and implications for therapeutic development.

RGS2 regulation of cellular functions

RGS proteins present differential selectivity towards Gα subtypes. Several RGS proteins can bind and act as GAPs for $G\alpha_q$, $G\alpha_{i/o}$ and $G\alpha_{12/13}$ with similar affinity (reviewed in e.g. (Zhang and Mende, 2011)). While several other RGS proteins are efficient GAPs for $G\alpha_q$, RGS2 is unique in that it is selective for $G\alpha_q$ over any other Gα subunit. In fact, early studies demonstrated that RGS2 is able to bind and act as a GAP only on $G\alpha_q$ *in vitro* (Heximer et al., 1997). In addition, RGS2 shows much greater potency than the closely related RGS4 in inhibiting $G\alpha_q$-mediated Phospholipase C (PLC) activation, indicating that RGS2 is not only selective for $G\alpha_q$, but a more potent GAP for $G\alpha_q$ than its closely related family members. A few later studies were able to demonstrate GAP activity towards $G\alpha_i$ in reconstituted membrane systems (Cladman and Chidiac, 2002; Ingi et al., 1998). RGS2 has also been shown to regulate $G\alpha_i$ signaling in cardiomyocytes (Chakir et al., 2011), however direct GAP activity towards $G\alpha_i$ or any other class of Gα has yet to be demonstrated for RGS2 *in vitro*. The molecular basis for the selectivity of RGS2 for $G\alpha_q$ remained poorly understood until the crystal structure of RGS2 in complex with $G\alpha_q$ was determined (Nance et al., 2013). It showed that RGS2 adopts a unique conformational pose compared to that of published RGS-$G\alpha_{i/o}$ complexes. RGS2 forms extensive

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interactions with the $G\alpha_q$ α-helical domain, a stabilizing network of interactions that may explain the increased affinity of RGS2 for $G\alpha_q$ compared to that of RGS4 (Nance et al., 2013).

Although it contains no additional protein domains apart from the RGS domain, several non-GAP functions have been attributed to RGS2. These functions are mediated by the ability of RGS2 to interact with several proteins, apart from $G\alpha_q$. The interactions identified thus far, as well as their functional consequences, are summarized in **Table 1**. Although RGS2 does not act as a GAP on $G\alpha_s$, it can inhibit Gs-mediated signaling through a direct interaction with certain isoforms of adenylate cyclase (AC), the enzyme downstream of $G\alpha_s$ that is responsible for the production of the second messenger cAMP. Specifically, RGS2 interacts with, and inhibits, AC III, the predominant isoform in the olfactory system, as well as the cardiac isoforms AC V and VI (Sinnarajah et al., 2001). This interaction is mediated through the N-terminal region of RGS2 and unrelated to the actions of the RGS domain. This was demonstrated by the inability of RGS2 lacking the first 19 residues to either bind AC V or to suppress Gs-mediated signaling (Salim et al., 2003).

Apart from the interaction with AC, RGS2 also interacts with several GPCRs directly. Through this action, RGS2 exerts further selectivity for certain Gq-coupled receptors over others. Early studies identified an interaction with the third intracellular loop of the Gq-coupled M1 muscarinic acetylcholine receptor, which was specific for RGS2 over the closely related RGS16 (Bernstein et al., 2004). This interaction was also selective for the M1 receptor over the other muscarinic receptors (M2-5). Recently, through studies using bioluminescence resonance energy transfer (BRET) in cells, RGS2 was shown to form a complex with the protease-activated receptor, PAR-1 as well (Kim and Ghil, 2020). This interaction seems dependent on concurrent interaction with $G\alpha_q$ and thus, may be dependent on activation state of the receptor. Furthermore,

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RGS2 has been suggested to interact with, and selectively enhance inhibition of signaling through, the Angiotensin II type 1 receptor (AT1R) (Matsuzaki et al., 2011). This study did not directly demonstrate binding of RGS2 to AT1R, however it demonstrated that the RGS2 N-terminus is an important determinant of the magnitude of suppression of AT1 Gq-mediated signaling. Thus, the N-terminal region of RGS2 appears to serve an important function in mediating protein-protein interactions with receptors, in addition to AC. It is also a key region for determining RGS2 protein stability as well as subcellular localization as described below.

An additional example of a non-canonical function of RGS2 is its ability to suppress total protein synthesis, through a direct interaction with the translation initiation factor EIF2ε (Nguyen et al., 2009). This action is mediated through a stretch in RGS2, partly overlapping with the RGS domain, but unrelated to GAP activity. The rate of total protein synthesis is directly involved in cell proliferation and growth, which can be induced by activation of GPCRs. RGS2 can suppress hypertrophy in neonatal rat cardiomyocytes, solely through its ability to suppress protein synthesis. Re-introduction of only the fragment of RGS2 responsible for this is enough to reverse hypertrophy (Chidiac et al., 2014). Altogether, although RGS2 is a seemingly simple protein, it is capable of several other functions in addition to acting as a GAP for Gα. These functions are mediated by protein-protein interactions, the majority of which occur through the N-terminal unstructured domain.

Regulation of RGS2 protein expression and activity

Activity and expression of RGS proteins, including RGS2, is tightly spatially and temporally regulated. This can be achieved through transcriptional, epigenetic and posttranscriptional mechanisms. A key regulatory mechanism controlling RGS2 protein levels is

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through rapid and constitutive degradation by the ubiquitin-proteasomal system (UPS). This system, critical for cell proliferation, differentiation, and survival, consists of a vast number of enzymes that couple a chain of ubiquitin molecules onto proteins to mark them for degradation by the 26S proteasome (Hershko and Ciechanover, 1998). Among these enzymes, the large family of E3 ligases (>600 known to date) recognizes substrates for ubiquitination and subsequent degradation through the 26S proteasome. In transfected cells, RGS2 has a protein half-life of ~20 min. Treatment of cells with the proteasome inhibitor MG-132 completely stabilizes RGS2 protein levels, indicating that proteasomal degradation plays an important role in its regulation (Sjögren et al., 2012). Selectively inhibiting RGS2 protein degradation could therefore be a viable strategy in disease states associated with low RGS2 protein levels, described in subsequent sections, and efforts have been made to identify the molecular machinery responsible for RGS2 protein degradation. RGS4 and RGS5, closely related to RGS2, are targeted for proteasomal degradation through the N-end rule pathway, where destabilizing residues at the very N-terminus of the protein serves as a recognition signal, or degron, for their cognate E3 ligase (Lee et al., 2005). RGS2 has also been proposed as an N-end rule substrate, where Gln^2 is acetylated to create a degron for the E3 ligase (Park et al., 2015). This model is supported by the finding that a Gln to Leu mutation in RGS2 (Q2L), identified in a hypertensive cohort, demonstrated enhanced proteasomal degradation (Bodenstein et al., 2007; Yang et al., 2005). This mutation would make RGS2 a direct target for the N-end rule pathway, as Leucine is a primary destabilizing residue, whereas Glutamine is a secondary destabilizing residue, requiring deamidation mediated by Glutamine-Specific N-Terminal Amidase (Ntaq1) to enter the N-end rule pathway (Sjögren and Neubig, 2010; Wang et al., 2009). However, later studies have

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failed to confirm the N-end rule pathway model of RGS2 protein degradation (Kanai et al., 2017; Sjögren et al., 2015).

An alternative model for RGS2 protein degradation was presented by our identification of a Cullin-RING ligase (CRL) that is able to degrade RGS2 in both transfected cells as well as in mouse cardiomyocytes (Sjögren et al., 2015). The protein that recognizes RGS2 within this complex is F-box only protein 44 (FBXO44). It is a member of the 69-member protein family of F-box proteins that have received increasing attention as drug targets in various diseases, ranging from several types of cancer to neurological disorders, such as Parkinson's Disease (Skaar et al., 2013; Wang et al., 2014). Whether these two models of RGS2 degradation – N-end rule pathway or F-box-mediated – are contradictory, or whether RGS2 is degraded through alternate pathways depending on cell type or context is yet to be determined. Regardless, the importance of the N-terminal region of RGS2 for protein stability is clear. RGS2 has four isoforms, resulting from alternative translation starting at Met^{1}, Met^{5}, Met^{16} and Met^{33}, respectively (Gu et al., 2008a) and the shorter variants (initiated at Met^{16} or Met^{33}) are protected from proteasomal degradation, further supporting the hypothesis that RGS2 is targeted for degradation through its N-terminus (Kanai et al., 2017).

Further studies into the intricate mechanisms regulating RGS2 protein levels and function have utilized non-synonymous single nucleotide polymorphisms (SNPs) identified through genomic studies. RGS proteins need to localize to the plasma membrane in order to act as GAPs for Gα. In the case of RGS2, this membrane targeting is mediated through a stretch in the N-terminal region. One SNP results in a R44H mutation, which was determined to completely block RGS2 plasma membrane targeting (Gu et al., 2008b). A subsequent study identified a second mutation, D40Y, resulting in similarly impaired plasma membrane targeting (Phan et al.,

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2017). Hence, apart from playing a key role in targeting RGS2 for protein degradation and protein-protein interactions, the N-terminus also plays a crucial role in targeting RGS2 to the plasma membrane. Additional SNPs have been found in both the coding region, as well as the 3'UTR of RGS2. In many cases, the functional effects on RGS2 protein levels and/or function have yet to be determined, but some have been extensively studied in terms of physiological impact. Some of these will be described in the following sections, in context of their possible clinical relevance.

In addition to interacting with, and modulating signaling through, certain GPCRs, as described above, RGS2 expression is also, in turn, regulated by GPCR activity. One example is the induction of RGS2 expression by long-acting β-adrenergic receptor agonists (LABA) used in asthma treatment (Holden et al., 2014). We discovered that RGS2 protein levels are increased by protein kinase C (PKC) activation occurring downstream of Gq-coupled GPCRs. These increased levels enhanced the ability of RGS2 to suppress G protein-mediated signaling and may serve as a general negative feedback loop for Gq-mediated signal transduction (Raveh et al., 2014). This also demonstrated that RGS2 activity is directly correlated with its expression levels. Whether the PKC-mediated increase in RGS2 protein levels is a result of direct phosphorylation is yet to be determined. In contrast to our findings, PKC phosphorylation of Ser^{46} was found to inhibit RGS2 GAP activity towards $G\alpha_{11}$ *in vitro* (Cunningham et al., 2001). Thus, the exact role of PKC regulation of RGS2 is still unclear and may depend on cellular context. Altogether, while much progress has been made in deciphering the mechanisms regulating RGS2 protein levels and activity, much is yet to be discovered.

RGS2 is a regulator of cardiovascular function

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The most well-defined physiological role for RGS2 is its regulation of the cardiovascular system. RGS2 is highly expressed in both the vasculature and heart, as well as in the kidney. Early studies in $RGS2^{-/-}$ mice shed light on the role of RGS2 in suppressing blood pressure, as they demonstrated both hypertension and prolonged responses to vasoconstrictor signaling by Gq-coupled GPCRs, such as AT1R and the purinergic receptor P2Y (Hercule et al., 2007; Heximer et al., 2003). However, regulation of blood pressure by RGS2 was later demonstrated to be more complex in nature. RGS2 is an effector of NO-mediated vasodilation in vascular smooth muscle cells (Obst et al., 2006; Sun et al., 2005; Tang et al., 2003). Phosphorylation of RGS2 on Ser^{46} and Ser^{60} by Protein Kinase G (PKG) induces translocation to the plasma membrane and modestly increases GAP activity of RGS2 towards $G\alpha_q$ (Osei-Owusu et al., 2007), suggesting that activation of NO-cGMP-PKG axis to induce vasodilation may be, at least in part, mediated by increased RGS2 function. Yet another study found that the regulation of blood pressure by RGS2 might not originate solely from the vasculature. In a cross-transplantation study, restoring RGS2 expression in the kidney was sufficient to restore normal blood pressure in $RGS2^{-/-}$ mice (Gurley et al., 2010). Although this study did not determine whether the effects of RGS2 expression originated from the kidney epithelium or vasculature, it indicates that the mechanisms mediating the effects of RGS2 on blood pressure homeostasis are more intricate than originally proposed.

Recent investigations have expanded on the role of RGS2 in the regulation of cardiovascular homeostasis. It appears that RGS2 plays an adaptive role in controlling uterine blood flow during pregnancy. $RGS2^{-/-}$ mice do not display increased blood flow normally associated with utero-placental perfusion during pregnancy (Koch et al., 2019). Furthermore, both $RGS2^{-/-}$ and $RGS5^{-/-}$ mice were more sensitive to α-adrenergic stimulation before pregnancy, with this sensitivity

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sustained through mid-pregnancy for RGS2$^{-/-}$ mice. Treatment of WT and RGS5$^{-/-}$ with the NO synthase inhibitor N$^{\omega}$-Nitro-L-arginine methyl ester (L-NAME) increased sensitivity to α-adrenergic stimulation to similar levels as RGS2$^{-/-}$ implying a possible mechanism for RGS2 dysfunction. This study reveals that RGS2 may play an important role in the regulation of vascular function during pregnancy (Koch et al., 2019). Further support for this was provided by a recent study demonstrating that RGS2 mRNA levels were reduced in the placenta during preeclampsia (Perschbacher et al., 2020). RGS2 was identified as a HDAC9-dependent (histone deacetylase 9) gene using immortalized human HTR8/SVneo trophoblasts. HDAC9 was reduced in human placentas affected by preeclampsia, suggesting a mechanism for the observed reduction in placental RGS2 mRNA levels. The same study also demonstrated that female mice with reduced RGS2 expression within the feto-placental unit displayed increased diastolic blood pressure and heart rate during the last week of gestation compared to WT mice, further supporting a protective role for RGS2 in preeclampsia (Perschbacher et al., 2020).

In addition to vascular regulation by RGS2, there is also ample evidence that RGS2 has a crucial role in regulating cardiac function through G protein-dependent and -independent mechanisms. While displaying no detrimental cardiac phenotype under normal conditions, RGS2$^{-/-}$ mice display augmented mortality, cardiac hypertrophy and cardiac fibrosis in response to pressure overload (Takimoto et al., 2009). In line with this observation, RNAi-mediated RGS2 knockdown in neonatal ventricular myocytes exacerbates phenylephrine- and endothelin-1-induced hypertrophy (Zhang et al., 2006). Additionally, *in vitro* overexpression of RGS2 inhibits cardiomyocyte hypertrophic effects induced by α1- or β-adrenergic receptor activation (Nunn et al., 2010). Although all of these effects could be attributed to the effects of RGS2 on Gq- and Gs-mediated signaling, a later study demonstrated that the 37-residue stretch in RGS2

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responsible for inhibiting *de novo* protein synthesis ($RGS2^{eb}$) inhibits cardiac hypertrophy comparably to full-length RGS2 (Lee et al., 2017).

While not directly focused on RGS2, a recent study found that vascular endothelial growth factor-B (VEGFB) reduced markers of hypertrophy in AngII-treated rat cardiomyocytes. The reduced AngII-mediated intracellular Ca^{2+} responses by VEGFB was determined to likely be due to stabilization of PKG and its downstream effectors, including RGS2. AngII treatment reduced RGS2 levels in cardiomyocytes, an effect which was partially abated upon co-treatment with VEGFB (Shen et al., 2018). Altogether, these data suggest that RGS2 may serve protective roles in cardiac function through multiple mechanisms and may be a potential drug target in cardiac, as well as vascular diseases associated with overactive GPCR signaling.

Data from human studies also support a role for RGS2 in the regulation of cardiovascular function. Several SNPs associated with reduced RGS2 protein levels and/or function have been linked to hypertension in several different ethnic groups (Riddle et al., 2006; Yang et al., 2005). One example is the SNP resulting in the Q2L mutation found in a Japanese hypertensive cohort. This mutation causes reduced expression of RGS2 due to enhanced proteasomal degradation (Bodenstein et al., 2007; Park et al., 2015; Phan et al., 2017) demonstrating that reduced RGS2 protein levels have an impact in humans as well as in murine models. Further support for the effect of altered RGS2 protein levels for cardiovascular functions is provided by evidence that RGS2 expression is increased in patients with Bartter's/Gitelman's syndrome, a disorder characterized by low blood pressure (Calo et al., 2004). Finally, low RGS2 protein levels have also been associated with non-responsiveness to antihypertensive treatment (Semplicini et al., 2010), suggesting RGS2 as a key regulator of blood pressure homeostasis in humans as well as in animal models.

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Roles for RGS2 in the CNS

RGS2 is widely expressed in the central nervous system (CNS), and has been linked to a number of CNS disorders, including anxiety and depression, as well as recently proposed links to Parkinson's and Alzheimer's disease. Although most studies thus far have only demonstrated association of RGS2 with disease states, with little to no mechanistic analysis, it is likely that RGS2 could also be a potential therapeutic target for the treatment of several CNS diseases.

The first evidence of a link between RGS2 and anxiety came from early studies in $RGS2^{-/-}$ mice, demonstrating enhanced anxiety as measured by the dark/light preference test (Oliveira-Dos-Santos et al., 2000). Subsequent research identified a quantitative trait locus in mice, containing the *RGS2* gene, that was associated with anxiety-related behavior (Yalcin et al., 2004). More recently, $RGS2^{-/-}$ mice were demonstrated to show enhanced fear learning, indicative of anxiety-like behaviors. There was also decreased neurotransmitter concentrations in $RGS2^{-/-}$ mice as well as lower expression of the serotonin (5-HT) receptor 5-HT_{2C}. In contrast, 5-HT_{2A} showed increased expression (Raab et al., 2018). In addition to the evidence provided by knockout models, mice heterozygous or homozygous for a SNP in the 3'UTR of the RGS2 gene, rs4606, which causes decreased RGS2 expression, show enhanced anxiety- and depression-like behaviors (Lifschytz et al., 2012). The rs4606 SNP was also associated with reduced expression of 5-HT_{1A} and 5-HT_{1B} receptors, specifically in the raphe nucleus, identifying a potential mechanism for RGS2-mediated anxiety-like behaviors (Lifschytz et al., 2012).

Following these initial studies, four SNPs associated with both panic disorder and agoraphobia were identified in the RGS2 gene in humans (Leygraf et al., 2006). An additional 9 SNPs were later associated with behavioral inhibition in children, which is a familial and

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developmental risk factor for anxiety disorders (Smoller et al., 2008). Four of these were also found to be associated with introversion in adults, another risk factor for anxiety disorders. Furthermore, two SNPs were associated with increased activity in the amygdala and left insular cortex, regions associated with anxiety, in response to an emotional test. In addition, rs4606 was deemed a risk factor for anxiety (Smoller et al., 2008). Further studies have solidified the association between rs4606 and various anxiety disorders in both children and adults, and suggest a relationship between low RGS2 levels and general anxiety disorders (Asselmann et al., 2018; Koenen et al., 2009; Stein et al., 2014).

In addition to anxiety-related disorders, there is also a tenuous link between RGS2 and Parkinson's Disease (PD) and Parkinson-like disease states. A phenotypic study suggested that genetic variations in the *RGS2* gene is associated with susceptibility to Parkinson-like extrapyramidal symptoms (EPS) induced by antipsychotic drugs. Again, rs4606 was found to be associated with Parkinson-like EPS in schizophrenic patients being treated with a typical antipsychotic drug alone or in combination with risperidone (Greenbaum et al., 2007). The G allele of rs4606 was later shown to be overrepresented in patients without EPS in a cohort of African-American and Caucasian schizophrenic patients, indicating a possible protective effect of this allele (Greenbaum et al., 2009). However, there are conflicting results from these human genetic screens on the impact of genetic variations in the RGS2 gene and Parkinson-like EPS in schizophrenia. Some studies have found no link between any haplotype of rs4606 and EPS or movement disorders (Al Hadithy et al., 2009; Bakker et al., 2012), while others have deemed the G allele to be detrimental (Higa et al., 2010). Thus, in humans, the impact of genetic variations in the *RGS2* gene on Parkinson-like EPS in schizophrenia remains unclear.

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Apart from human genetic screens, a few studies have provided *in vitro* mechanistic data on the possible involvement of RGS2 in PD. Mutations in leucine-rich repeat kinase 2 (LRRK2) are a common marker of PD. siRNA knock-down of RGS2 in LRRK2-expressing *C. elegans* increased the survivability of dopaminergic neurons. Follow-up experiments elucidated that the RGS domain and LRRK2 co-immunoprecipitate with each other even with PD-associated LRRK2 mutations. RGS2 decreases both the GTPase and kinase activity of LRRK2, resulting in an inhibition of LRRK2-dependent neurite shortening. It was also found that RGS2 expression is decreased in the striatum of mutant LRRK2 patients and sporadic PD patients compared to control, providing a clinical link between RGS2 and PD (Shen et al., 2018).

Dopamine is a central regulator of motor functions and reward, and plays a central role in the pathology of addiction, Schizophrenia and PD. A 2016 study, found that RGS2 negatively modulates dopamine D2 receptor (D2R) signaling in neuroblastoma N2A cells (Luessen et al., 2016). In addition, RGS2 knockdown was found to increase constitutive D2R internalization while preventing quinpirole-induced internalization. The latter effect is due to a tighter interaction between D2R and β-arrestin resulting in less β-arrestin dissociation from the membrane. While this study does not look into the implications of RGS2 in PD concerning these interactions, demonstrating that RGS2 directly alters dopamine signaling and receptor internalization provides strong justification for future studies (Luessen et al., 2016).

Finally, there is also a suggested association between RGS2 levels and the progression of Alzheimer's disease (AD). A genome-wide transcriptomic study identified lower RGS2 mRNA levels being correlated with increased sensitivity to Amyloid-$\beta_{1\text{-}42}$ treatment in cell culture. Accumulation of amyloid-β plaques is one of the primary markers of AD, and these results implicate that RGS2 may play a protective role against this hallmark of the disease. Furthermore,

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RGS2 mRNA levels were decreased in cells isolated from AD patients compared to healthy controls. In contrast to these results, however, lower RGS2 levels were also correlated with better results for the Mini Mental State Examination and AD Assessment Scale, two tests that indicate cognitive ability. These cognitive effects were attributed to enhanced muscarinic signaling, enhanced melatonin production, and enhanced EIF2Bε-mediated translation, all processes regulated by RGS2 (Hadar et al., 2016). Taken together, low RGS2 mRNA correlated with both detrimental and beneficial outcomes in AD and more studies are warranted to decipher the mechanistic basis for the role that RGS2 might play in the progression of this disease.

RGS2 as a potential cancer target

As described above, RGS2 can mediate effects on cellular functions through several different mechanisms in addition to being a negative regulator of G protein signaling. These additional functions may contribute to and be associated with a number of malignancies. Although the specific mechanisms by which RGS2 is involved in cancer progression is less understood than in the cardiovascular system, emerging evidence suggest an important regulatory role in several types of cancer, including breast, prostate, acute myeloid leukemia, bladder, ovarian, and colorectal cancer.

Breast cancer is the second deadliest cancer in the US (Siegel et al., 2019), with several subtypes, many of which lack effective treatments. A proposed role for RGS2 as a tumor suppressor has recently emerged. RGS2 is downregulated in breast cancer cells and human tumor samples, and overexpression of RGS2 can inhibit MCF-7 breast cancer cell growth (Lyu et al., 2015). In addition, both mRNA and protein levels of RGS2 were shown to be downregulated in breast invasive carcinoma of no special type (BIC-NST), the most common subtype of invasive

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breast cancer (Wang et al., 2018). This same study also demonstrated that patients with lower RGS2 expression levels had a significantly poorer overall survival rate (Wang et al., 2018). The mechanisms involved in RGS2 suppression and how RGS2, in turn, regulates breast cancer progression is still under investigation. One proposed regulatory mechanism is mediated through the deubiquitinating enzyme (DUB) monocyte chemotactic protein-induced protein 1 (MCPIP1) that was demonstrated to protect RGS2 from degradation in MCF-7 cells (Lyu et al., 2015). However, it is still uncertain whether this mechanism occurs endogenously in breast cancer, as these studies were performed using overexpression systems. Overall, these studies provide support for RGS2 as a tumor suppressor and a potential target for breast cancer treatment. However, other studies seem to contradict these data (Kelly et al., 2006a; Xie et al., 2009). Hence, the role of RGS2 as a breast cancer tumor suppressor warrants further investigation.

The role of RGS2 in prostate cancer seems to be complex and dependent on cancer stage. In clinical samples, RGS2 protein levels are downregulated by moderate hypoxia during primary prostate cancer development while it is upregulated in late stages (Linder et al., 2018). Therefore, RGS2 protein levels might be used as a prognostic maker to distinguish between primary and advanced patients. In addition, by analyzing a cohort of advance prostate cancer, a correlation was found between high RGS2 level and poor patient survival and metastasis in their studies (Linder et al., 2018). Furthermore, RGS2 knock-down in LNcap prostate cancer cells decreased migration and induced epithelial cell morphology and behavior (Linder et al., 2018). All these data indicate that RGS2 contributes to metastasis in androgen-sensitive prostate cancer. However, in the androgen-independent stage, RGS2 is proposed to play a tumor suppressor role. Microarray data demonstrated decreased RGS2 protein levels in prostate carcinoma (Wolff et al., 2012). The proposed mechanism for RGS2 downregulation is through hypermethylation of the

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RGS2 promotor region. Furthermore, increased RGS2 expression levels inhibits androgen-independent cell growth as well as tumor growth in a xenograft mouse model. This is consistent with microarray data showing that RGS2 is one of the most plausible candidates causing repressed androgen-independent prostate tumor growth (Jennbacken et al., 2009; Wolff et al., 2012). The inhibition of cell growth induced by RGS2 may be attributed to the observation that RGS2 can attenuate androgen-independent AR activity (Cao et al., 2006). However, the mechanism by which RGS2 exerts these effects is currently unknown, as GAP activity cannot explain the suppression. Further support for a protective role of RGS2 in androgen-independent prostate cancer is provided by the demonstration that overexpression of RGS2 can inhibit OXT-, TGFβ1- and EGF-induced PC3 cell migration (Caggia et al., 2019). In contrast, another group found no effect of RGS2 overexpression on thrombin-stimulated cell invasion in PC3 cells or another androgen-insensitive cell line, DU145 (Kelly et al., 2006b). Furthermore, a recent study showed that higher RGS2 protein levels were found to be prognostic for poor survival in castration-resistant prostate cancer and that *RGS2* gene expression levels positively correlated with AR expression and activity (Linder et al., 2020). Thus, the role of RGS2 in prostate cancer is complex, and may depend on the stage of cancer progression.

Apart from breast and prostate cancer, RGS2 has also been proposed as a tumor suppressor in Acute Myeloid Leukemia (AML). RGS2 was expressed at significantly lower levels in AML patients in comparison to normal bone marrow, especially in patients with the FLT3-ITD mutation (an internal tandem duplication in FMS-like tyrosine kinase 3), the most common mutation in AML (Schwable et al., 2005). In addition, RGS2 overexpression inhibits FLT3-ITD-induced cell proliferation and growth, and antagonizes differentiation blockade induced by this mutation, which is an important step for malignant transformation (Schwable et al., 2005). The

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mechanism by which RGS2 is downregulated or by which mechanism RGS2 achieves the effects in AML is not yet defined.

Repression of RGS2 has been proposed to be associated with other less common cancer types such as bladder cancer, ovarian cancer and colorectal cancer as well. RGS2 expression levels was found to be significantly lower in tumor tissues than in matched normal tissues, and low RGS2 expression was correlated with reduced overall survival in patients with bladder cancer (Ying et al., 2015). RGS2 suppression could be attributed to a higher percentage of promotor hypermethylation because of increased expression of UHRF1, a regulator of DNA methylation (Ying et al., 2015). Epigenetic suppression of RGS2 expression has also been observed in chemoresistant ovarian cancer. RGS2 expression level is slightly lower in ovarian cancer cells and overexpression of RGS2 can inhibit LPA-mediated downstream signaling, which is important for ovarian cancer cell growth (Hurst et al., 2009). In this instance, RGS2 expression is suppressed in chemoresistant ovarian cancer cells, in part due to accumulation of histone deacetylase (HDACs) and DNMT at the RGS2 promotor region (Cacan, 2017). However, it is still unknown if downregulation of RGS2 is the causation for the progression and chemoresistance in ovarian cancer. Finally, low RGS2 mRNA and protein levels have been associated with poor survival in stage II and III colorectal cancer (Jiang et al., 2010). This study only demonstrated association, without dissecting potential mechanisms for RGS2 suppression, or how RGS2 would suppress colorectal cancer progression. However, it is yet another example of low RGS2 levels correlating with disease progression.

While the above examples demonstrate downregulation of RGS2 and a possible tumor suppressor role, this may not hold true for all cancer types. For instance, upregulation of RGS2 is related to poor survival in lung adenocarcinoma patients (Yin et al., 2016). In contrast,

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downregulation of RGS2 is associated with increased invasion and metastasis of human non-small cell lung cancer cells caused by loss of Med1/TRAP220 (Kim et al., 2012). These studies emphasize that RGS2 may play different roles depending on the cancer subtype, even in the same tissue. Another example of where overexpression of RGS2 may promote cancer progression is demonstrated by Mantle Cell Lymphoma (MCL). In a cDNA array study, RGS2 was found as one of four genes suggested to promote tumor progression in both the blastoid variant and common MCL. (Zhu et al., 2002). Additionally, RGS2, among several signal transduction genes in the Ras, MAPK and PI3K pathways, is overexpressed in both primary and metastatic fibrolamellar carcinoma tumors (Kannangai et al., 2007). Collectively, these studies demonstrate, by association, that RGS2 expression levels can be one of the factors promoting tumorigenesis, however further mechanistic analysis is warranted to determine whether altered RGS2 expression is a cause or effect in these cancers.

Although progress has been made on investigating the association between altered RGS2 expression and cancer progression, there still exists a large gap in knowledge concerning the molecular mechanisms underlying changed RGS2 expression levels. Furthermore, in the majority of cancers, we do not know whether the change in RGS2 mRNA and protein levels is a driver in cancer progression, or if it is a result of altering other important tumor suppressors or oncogenes. Finally, the mechanisms by which RGS2 may alter proliferation, migration or invasion of cancer cells are currently unknown. Increased knowledge of all of these concepts will enhance our understanding of the diverse phenotypes related with RGS2 in different cancer types. Dissection of the mechanisms involved will aid in future cancer therapy development.

Additional clinical implications

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Apart from the clinical implications described above, RGS2 may play a role in several other areas. Like in the cardiovascular system, airway constriction is also regulated through Gq-coupled activation. RGS2 has been proposed to play a significant role in suppressing Gq signaling, and subsequent inflammatory responses and airway remodeling. RGS2 expression levels are reduced in asthmatic patients (Jiang et al., 2015) and overexpression of RGS2 reduces intracellular free calcium flux elicited by histamine, methacholine, leukotrienes, and other Gq-coupled spasmogens in primary human airway smooth muscle (ASM) cells. In line with this, $RGS2^{-/-}$ mice display enhanced ASM contractility (Holden et al., 2011; Xie et al., 2012). In addition, the standard of care treatment with corticosteroids and LABA synergistically induce RGS2 expression in both the human bronchial epithelial cell line BEAS-2B as well as primary bronchial epithelial cells (Holden et al., 2014), possibly explaining the beneficial effects of the combination treatment compared to corticosteroid treatment alone. This is further supported by the fact that protection against Gq-mediated increases in intracellular free calcium, following LABA plus corticosteroid treatment, is dependent on RGS2 (Holden et al., 2011). RGS2 also plays a role in inflammatory responses elicited by Gq. In a model of airway inflammation induced by inhaled lipopolysaccharide (LPS), $RGS2^{-/-}$ mice displayed increased airway hyperreactivity in response to the muscarinic receptor agonist methacholine, compared to WT mice (George et al., 2018). $RGS2^{-/-}$ mice also displayed enhanced airway hyperresponsiveness in a model of house dust mite-induced airway inflammation (George et al., 2017). In addition, recruitment of inflammatory cells, neutrophils and eosinophils to the bronchoalveolar lavage fluid were significantly increased in $RGS2^{-/-}$ mice (George et al., 2017). Altogether, these data suggest that RGS2 plays a protective against airway hyperreactivity and may be a promising

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target in suppressing aberrant activation of Gq signaling and downstream inflammatory responses in asthma.

Additional suggested physiological functions for RGS2 include, but are not limited to, kidney fibrosis progression regulation (Jang et al., 2014), brown adipose tissue function and differentiation (Klepac et al., 2019), as well as serving as a key regulator of pancreatic β-cell function (Dong et al., 2017). In a 2017 study, $RGS2^{-/-}$ mice were found to have enhanced β-cell apoptosis and dysregulated insulin secretion in response to glucose challenge. Based on these and additional data in β-cells and isolated β-islets, the authors suggested that RGS2 serves a protective role against β-cell loss and may be a promising drug target in the treatment of both type 1 and type 2 diabetes (Dong et al., 2017). Although the data on these additional functions are limited to only a few, or even a single publication, the common theme among these studies is that low RGS2 protein levels, and thereby function, are detrimental to physiological function.

Future perspectives for therapeutic targeting

From all the aforementioned studies it seems that RGS2 may be a promising drug target with broad clinical implications. RGS2 protein levels and/or function is altered in a wide range of pathologies, and in the majority of scenarios low RGS2 protein levels correlate with detrimental effects on physiological function. RGS proteins have received increasing attention as potential drug targets in the past couple of decades. As crucial regulators of GPCR signaling, they represent a novel point of intervention for targeting G protein-mediated responses. GPCRs are the primary target of a large number of clinically used drugs. Many of these are associated with side effects, due to the widespread distribution of any given GPCR, and the fact that a single receptor can initiate a wide range of G protein-dependent and –independent signaling

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cascades. Thus, in recent years efforts have been made to develop allosteric modulators or biased agonists that would direct signaling through the receptor in a more fine-tuned manner. It is feasible to imagine that an RGS protein modulator would serve a similar function. Taking into account G protein selectivity and tissue distribution, a specific RGS protein would differentially affect signaling cascades initiated by a GPCR. Furthermore, in the absence of receptor activation, an RGS modulator would not have an effect on G protein signaling. Taken together, RGS proteins could have big therapeutic potential, serving similar roles as allosteric modulators at GPCRs.

Identifying viable small molecule drug leads targeting RGS2, as for all RGS proteins, represents a challenge, in particular when the goal is to enhance function. RGS2 proteins are not highly amenable to small molecule binding and although several RGS protein inhibitors have been developed (Blazer et al., 2010; Blazer et al., 2011; Bodle et al., 2017; Bodle et al., 2018; Roman et al., 2007), enhancing function by means of a small molecule would be even more difficult. We have applied unbiased screening approaches to identify small molecules that increase RGS2 protein levels, and thereby function. Through these efforts, we identified digoxin and other cardiotonic steroids (CTS) to selectively enhance RGS2 protein levels, by extending protein half-life (Sjögren et al., 2012). Interestingly, just a modest increase in RGS2 protein levels (2-3 fold) was sufficient to inhibit G protein-mediated signaling both *in vitro* and *in vivo* and was cardioprotective in a mouse model of cardiac injury (Sjögren et al., 2016). These data served as proof-of-concept that increased RGS2 protein levels correlate with increased protein function, and mechanisms that mediate the regulation of RGS2 steady-state level could be targeted as a therapy in a wide range of pathologies. As more of these mechanisms are revealed, the potential for RGS2 as an emerging drug target grows.

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Author contributions

Participated in research design: McNabb, Zhang, Sjögren

Conducted experiments: N/A

Contributed new reagents or analytic tools: N/A

Performed data analysis: McNabb, Zhang, Sjögren

Wrote or contributed to the writing of the manuscript: McNabb, Zhang, Sjögren

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Tables

Table 1. Summary of RGS2 protein-protein interactions. Listed are confirmed interactions for RGS2 and their functional consequences. This list is not comprehensive, and more interactions are likely to be found in the future.

Interacting protein		Region of interaction	Functional consequence	Reference(s)
Gα subunits:	$G\alpha_q$	RGS domain; high affinity	GAP activity	e.g. (Ingi et al., 1998; Nance et al., 2013)
	$G\alpha_{i1}$	RGS domain; low affinity	GAP activity	e.g. (Ingi et al., 1998)
	$G\alpha_s$	RGS domain	Modulate signaling; no GAP activity	(Roy et al., 2006)
GPCRs:	Angiotensin II type 1 receptor (AT1R)	N-terminus	Inhibition of AT1R signaling	(Matsuzaki et al., 2011)
	Protease-activated receptor 1 (PAR1)	N-terminus; third intracellular loop of PAR1; Gα dependent	Inhibition of PAR1 signaling	(Ghil et al., 2014)
	Protease-activated receptor 4 (PAR4)	N-terminus; third intracellular loop of PAR4; Gα dependent	Inhibition of PAR4 signaling	(Kim and Ghil, 2020)
	Muscarinic M1 receptor (M1R)	N-terminus; third intracellular loop of M1R	Inhibition of M1R signaling.	(Bernstein et al., 2004)
	α1-Adrenoreceptor (α1AR)	N-terminus; third intracellular loop of α1AR	Inhibition of α1AR signaling.	(Hague et al., 2005)
	β2-Adrenoreceptor (β2AR)	N-terminus; third intracellular loop of β2AR	Inhibition of β2AR signaling.	(Roy et al., 2006)
	Melanin-concentrating hormone receptor 1 (MCH1R)	Residues 28-80	Inhibition of MCH1R signaling.	(Miyamoto-Matsubara et al., 2010)
Other:	Adenylate cyclase III (AC III)	N-terminus; C1 region of AC III	Inhibits cAMP production.	(Sinnarajah et al., 2001)
	Adenylate cyclase V (AC V)	N-terminus; C1 region of AC V	Inhibits cAMP production.	(Sinnarajah et al., 2001)
	Adenylate cyclase VI (AC VI)	N-terminus; C1 region of AC V	Inhibits cAMP production.	(Sinnarajah et al., 2001)
	eIF2Bε	Residues 79-115	Inhibits protein synthesis.	(Wang et al., 2018)
	cGMP-dependent protein kinase I-α (PKGI-α)	RGS2 phosphorylation	Inhibits RGS2 degradation; promotes RGS2 membrane localization.	(Osei-Owusu et al., 2007; Tang et al., 2003)
	Protein kinase C (PKC)	Phosphorylation on Ser^{46}	Inhibits GAP activity *in vitro*	(Cunningham et al., 2001)
	TRPV6	N-terminus	Modulates channel activity	(Schoeber et al., 2006)

leucine rich repeat kinase 2 (LRRK2)	Undetermined	Control of neuronal process length, protective against neuronal toxicity.	(Dusonchet et al., 2014)
Nek7	Undetermined	Mitotic spindle organization.	(de Souza et al., 2015)
FBXO44	N-terminus	E3 ligase; promotes RGS2 protein degradation	(Sjögren et al., 2015)
Teb4	N-terminus	E3 ligase; promotes RGS2 protein degradation	(Park et al., 2015)

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Figure legends

Figure 1. Summary of RGS2 protein physiological functions. RGS2 is widely expressed in various tissues and there is support for a role in regulating diverse physiological functions, ranging from vaso- and bronchoconstriction, cardiac hypertrophy, CNS functions, as well as insulin secretion and β-cell physiology. Structure adapted from 4EKD, RGS2 in complex with $G\alpha_q$ (Nance et al., 2013).

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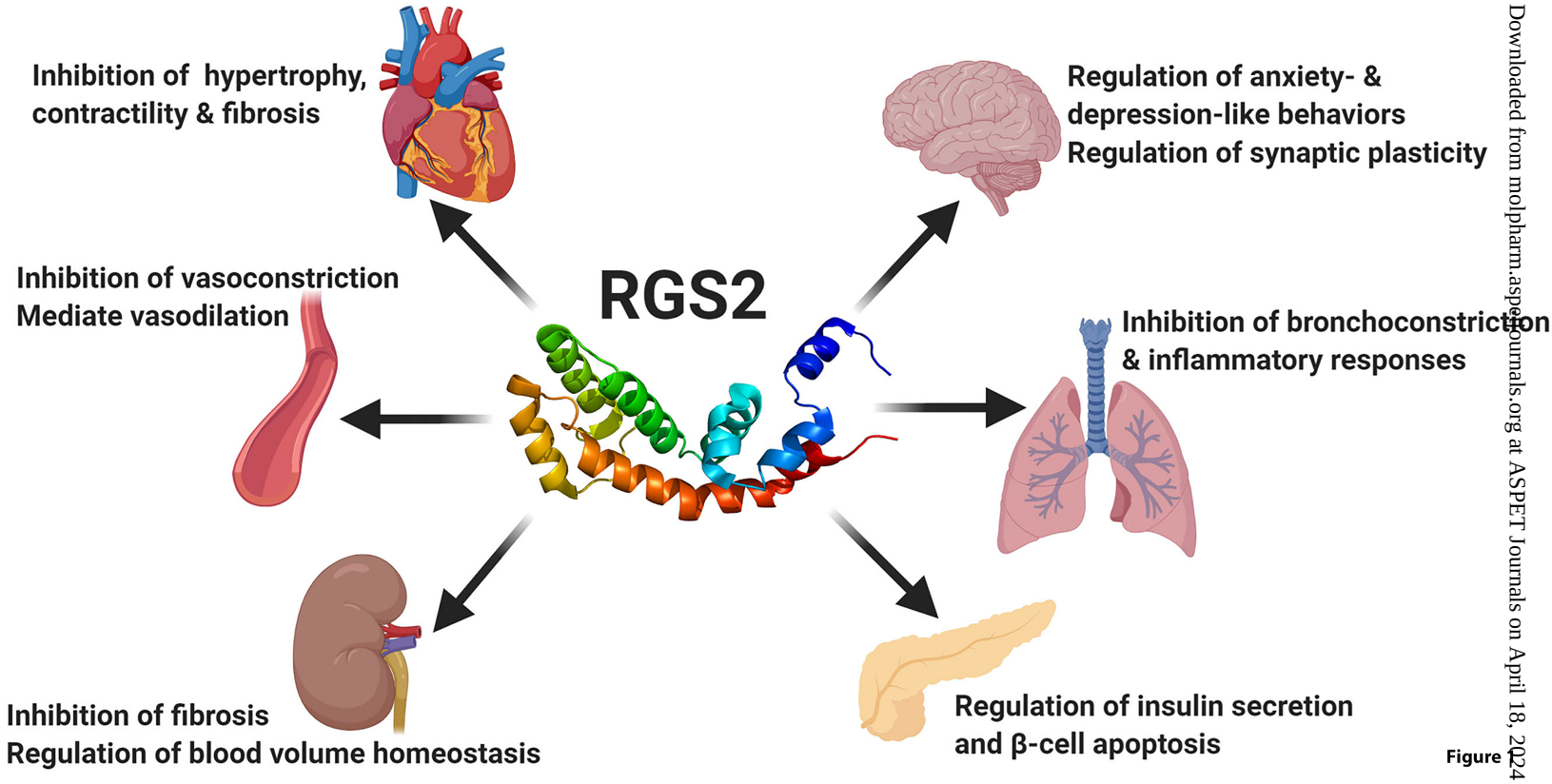

Figure 1