Review Article

Theme: Heterotrimeric G Protein-based Drug Development: Beyond Simple Receptor Ligands

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RGS6 as a Novel Therapeutic Target in CNS Diseases and Cancer

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Abstract. Regulator of G protein signaling (RGS) proteins are gatekeepers regulating the cellular responses induced by G protein-coupled receptor (GPCR)-mediated activation of heterotrimeric G proteins. Specifically, RGS proteins determine the magnitude and duration of GPCR signaling by acting as a GTPase-activating protein for $G\alpha$ subunits, an activity facilitated by their semiconserved RGS domain. The R7 subfamily of RGS proteins is distinguished by two unique domains, DEP/DHEX and GGL, which mediate membrane targeting and stability of these proteins. RGS6, a member of the R7 subfamily, has been shown to specifically modulate $G\alpha_{i/o}$ protein activity which is critically important in the central nervous system (CNS) for neuronal responses to a wide array of neurotransmitters. As such, RGS6 has been implicated in several CNS pathologies associated with altered neurotransmission, including the following: alcoholism, anxiety/depression, and Parkinson's disease. In addition, unlike other members of the R7 subfamily, RGS6 has been shown to regulate G protein-independent signaling mechanisms which appear to promote both apoptotic and growth-suppressive pathways that are important in its tumor suppressor function in breast and possibly other tissues. Further highlighting the importance of RGS6 as a target in cancer, RGS6 mediates the chemotherapeutic actions of doxorubicin and blocks reticular activating system (Ras)-induced cellular transformation by promoting degradation of DNA (cytosine-5)-methyltransferase 1 (DNMT1) to prevent its silencing of pro-apoptotic and tumor suppressor genes. Together, these findings demonstrate the critical role of RGS6 in regulating both G protein-dependent CNS pathology and G protein-independent cancer pathology implicating RGS6 as a novel therapeutic target.

KEY WORDS: alcoholism; depression; doxorubicin; Parkinson's disease; RGS protein.

INTRODUCTION

G protein-coupled receptors (GPCRs) are involved in virtually every known physiological process, and dysfunction in their signaling is linked to many human diseases. GPCRs become active in response to extracellular agonist binding which induces conformational changes in the receptor promoting its association with heterotrimeric G proteins (1), consisting of three functional subunits: the GDP/GTP-binding α subunit, and the β and γ subunits. Agonist-activated GPCRs function as GTP exchange factors (GEFs) for $G\alpha$ subunits, promoting exchange of GDP for GTP and resulting in $G\alpha$ subunit activation and dissociation from $G\beta\gamma$ subunits, with both $G\alpha$ -GTP and $G\beta\gamma$ activating downstream signaling

pathways (2). Four families of $G\alpha$ subunits, $G\alpha_i$, $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12}$, that exhibit selectivity in terms of their coupling to GPCRs and their downstream signaling actions, contribute in part to the signaling specificity of different GPCRs. The intrinsic GTPase activity of $G\alpha$ subunits is responsible for hydrolysis of GTP, reformation of inactive $G\alpha$ -GDP subunits and their reassociation with $G\beta\gamma$, effectively terminating both $G\alpha$ and $G\beta\gamma$ signaling. Regulator of G protein signaling (RGS) proteins act as GTPase-activating proteins (GAPs) for $G\alpha$ subunits by stabilizing the transition state of the GTP hydrolysis reaction by $G\alpha$ subunits. Therefore, RGS proteins play a critical role in regulating the duration and magnitude of signaling initiated by GPCRs by serving as gatekeepers of signaling mediated by G protein $G\alpha$ and $G\beta\gamma$ subunits (3–6) (Fig. 1).

There are 20 canonical mammalian RGS proteins that have been divided into four subfamilies based upon sequence homology and protein domain structure. RGS6 is a member of the R7 subfamily (RGS6, RGS7, RGS9, RGS11) of RGS proteins that shares two unique domains outside of the RGS domain (common to all RGS proteins): the disheveled EGL-10, pleckstrin homology (DEP)/DEP helical extension (DHEX) domain and the G gamma subunit-like (GGL) domain (Fig. 2). Together, these three domains modulate

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Fig. 1. Regulation of G protein-coupled receptor (GPCR) signaling by regulator of G protein signaling (RGS) proteins. RGS proteins act as GTPase-activating proteins (GAPs) for specific G α subunits and thereby function to terminate GPCR signaling

RGS6 protein stability, localization, and function. In considering RGS6 protein stability, interaction of the GGL domain and the atypical G β_5 subunit, G β_5 , is a general requirement for stabilization of the whole R7 protein subfamily (8–10). As such, genetic ablation of the G β_5 gene (*GNB5*) is correlated with the loss of the R7 protein subfamily in the retina and striatum (11). However, the ability of G β_5 to stabilize RGS6 may not be solely dependent on its interaction with the GGL domain, but may require a direct interaction with its DEP/DHEX domain as well. In evidence of this, G β_5 has also been shown (*via* crystal structure and pull-down experiments) to interact with the DEP/DHEX domain of the R7 family members RGS7 and RGS9, and mutation of G β_5 residues mediating this interaction leads to the instability of both RGS proteins (12–14). In addition to promoting protein stability, both the GGL and DEP/DHEX domains are also important for modulating RGS6 cellular localization. Experiments in which COS-7 cells were transfected with GFP-tagged RGS6 splice variants demonstrated that the GGL domain promotes cytoplasmic retention of RGS6. However, when the GGL domain is lost due to alternative splicing (–GGL variants, Figs. 2 and 3), or when G β_5 is overexpressed to generate RGS6:G β_5 complexes, GFP-tagged RGS6 moves into the nucleus (7). Similarly, the DEP/DHEX domain also regulates cytoplasmic-nuclear shuttling of RGS6. Indeed, further experiments looking at the subcellular localization GFP-tagged RGS6 protein variants in COS-7 cells demonstrated that the RGS6 splice variants containing the DEP/DHEX domain (RGS6 long (RGS6L) variants, Figs. 2 and 3) were largely cytoplasmic, whereas those lacking the domain (RGS6 short (RGS6S) variants, Figs. 2 and 3) were primarily nuclear (7). It is believed that this shuttling may in part be due to a DEP/DHEX-mediated interaction of RGS6 with R7 family-binding protein (R7BP) as it has been shown that R7BP is reversibly palmitoylated promoting either a membrane (palmitoylated) or nuclear (depalmitoylated) distribution of another R7 family member, RGS7 (15). This differential subcellular localization of RGS6 appears to be functionally relevant as it can also be seen in native tissues. For example, immunohistochemical analysis of RGS6 protein localization in the mouse cerebellum, using an antibody that the Fisher

laboratory generated against the N-terminal protein domain, common among all RGS6L isoforms, demonstrated that RGS6L has distinct cytoplasmic and nuclear localization patterns (7). In further support of the functional relevance of this differential subcellular localization, other R7 family members, in particular RGS7 and RGS9, as well as G β_5 have also been shown to have both distinct cytoplasmic and nuclear localization patterns (16–19). Finally, in terms of RGS6 function in negatively regulating heterotrimeric G protein signaling, the RGS domain is responsible for the GAP activity of RGS6, and other RGS proteins, and allows it to negatively regulate G $\alpha_{i/o}$ proteins (20). RGS6 specific modulation of G $\alpha_{i/o}$ protein activity has been implicated in the regulation of several disease states, particularly in the central nervous system (CNS), including the following: alcoholism (21), anxiety/depression (22), Parkinson's disease (23), and potentially Alzheimer's disease (24), schizophrenia (25), and vision (26). However, RGS6 is also unique in that it remains the only member of the R7 protein family that has been demonstrated to regulate G protein-independent pathways, as evidenced by its compelling pro-apoptotic and tumor suppressor actions in cancer (27–30).

Potentially key to RGS6's G protein-independent signaling, as well as its modulation of G protein signaling, are previously unidentified domains present in a subset of RGS6 proteins. These domains may arise *via* alternative splicing of RGS6 messenger RNA (mRNA) transcripts. In support of this idea, when the Fisher laboratory first cloned RGS6 from a Marathon-ready human brain cDNA library (brain tissue is where RGS6 is most highly expressed at the mRNA (31) and protein level (Fisher Laboratory, unpublished)), they described 36 distinct isoforms that could arise through complex splicing of two primary RGS6 transcripts (7) (Fig. 3). These 36 distinct splice forms are predicted to produce 18 long isoforms (RGS6L) ranging from ~49 to 56 kDa in size and 18 short isoforms (RGS6S) ranging from 32 to 40 kDa. While the various RGS6L and RGS6S splice forms are largely similar in sequence, making it difficult to develop antibodies to confirm their individual existence and determine their individual function, the Fisher laboratory has had some success in characterizing the proteins resulting from

Fig. 2. Predicted protein structure of human RGS6 proteins. There are predicted to be numerous RGS6 protein isoforms that differ in length due to the following: inclusion or exclusion of the disheveled EGL-10, pleckstrin homology (DEP) domain at their N-terminus, inclusion or exclusion of a complete G gamma subunit-like (GGL) domain, and in the inclusion of one of seven distinct C-termini. RGS6 proteins with either the long or the short N-terminus are labeled as RGS6L or RGS6S, respectively. The C-terminal domains are labeled as α, β, γ, δ, ε, η, and ζ. The α and β C-termini exist in two forms, either with (α1 and β1) or without (α2 and β2); an 18 amino acid sequence encoded by exon 18 (grey square) of the RGS6 gene. Finally, proteins that lack the GGL domain are designated as -GGL proteins. Amino acid numbers are included to specify where key regions of the protein begin and end. Image adapted from reference (7)

several of these splice variants. As mentioned earlier, characterization of the differential subcellular localization for multiple GFP-tagged RGS6 protein isoforms in COS-7 cells demonstrated that an alteration in RGS6 protein structure can dictate whether the protein is primarily localized in the cytoplasm (RGS6L and +GGL protein isoforms) or nucleus (RGS6S and -GGL protein isoforms), suggesting that alternatively spliced RGS6 transcripts may result in proteins with unique functions, and indeed such differential localization of RGS6L was also

seen in native tissues (7, 32). The Fisher lab has also demonstrated using western blot that certain tissues express multiple distinct RGS6 protein isoforms natively. For example, the brain expresses at least two distinct RGS6 isoforms that are larger (~61 and 69 kDa) than ubiquitously expressed smaller forms of the protein (21, 33). Interestingly, western blot analysis of brain tissue lysates using the antibody against the N-terminal protein domain, common to all RGS6L proteins, reveals a broad band of RGS6 immunoreactivity which could be explained by the presence of multiple RGS6L

Fig. 3. Diagram of the complex splicing of human RGS6 pre-mRNA to generate 36 splice variants. Two primary transcripts encode the 5'-splice forms of RGS6; the AUG-1 start site produces a transcript that encodes the RGS6L forms of the protein while the AUG-2 start site produces a transcript that encodes the RGS6S forms of the protein. Retention or removal of exon 13 (first pink square) generates transcripts that encode for proteins containing or lacking a complete GGL domain, respectively. 3'-splicing generates transcripts containing seven distinct 3' exons. RGS6 α and β transcripts exist in two forms that arise from either the retention (α1 and β1) or removal (α2 and β2) of exon 18 (second pink square). Image adapted from reference (7)

isoforms with different C-terminal domains and with or without complete GGL domains (22, 34). The functions for these RGS6 variants and how they all arise (either through protein modification or additional RNA splicing) are unknown.

RGS6 IN CNS DISEASES

Alcohol Use Disorders

Approximately 12% of the US population suffers from alcoholism causing a substantial annual economic burden (~\$223.5 billion). In light of these statistics, researchers have sought to identify and understand the underlying mechanisms of alcohol dependence, but have been met with only limited success. As a result, there are few therapeutic options available to reduce alcohol cravings and withdrawal symptoms and there are no drugs that have been approved to prevent/treat alcohol-related organ damage. Part of the problem is that alcohol does not have a specific molecular target in the brain, but instead induces neuronal alterations in the mesolimbic pathway (implicated in drug addiction (35–38)) by both inhibiting *N*-methyl-D-aspartate (NMDA) receptor activity and enhancing gamma-aminobutyric acid B (GABA_B) receptor activity (39).

Although alcohol disrupts mesolimbic neuronal signaling *via* multiple mechanisms, the end result is an alteration in neurotransmitter release. As the majority of neurotransmitters in the mesolimbic pathway (e.g., dopamine (DA), GABA, opioids, and serotonin (5-HT)) interact with GPCRs, G protein-dependent signaling may offer a therapeutic target in the treatment of alcohol abuse. With this in mind, multiple drugs targeting these neurotransmitter receptors have been recommended for the treatment of alcoholism (40–42). One such drug, baclofen, a GABA_BR agonist, has been approved in Europe as a treatment for alcohol withdrawal symptoms and cravings (43–45). However, despite the positive effects of baclofen in the treatment of alcohol abuse, its use remains limited as it compounds both the muscle relaxant and sedative properties of alcohol.

In light of the fact that baclofen-mediated modulation of the GABA_BR is a viable treatment for alcoholism, RGS6 also became a protein of interest, as previous research had demonstrated its ability to negatively regulate GABA_BR signaling in the cerebellum (33). In addition, there was also evidence to suggest that RGS6 was capable of regulating the signaling of other GPCRs, such as 5-HT_{1A}Rs and μ -opioid receptors (22, 46), which had already been identified as potential therapeutic targets in the treatment of alcoholism (40, 42). Both immunohistochemical and western blot studies in wild type (RGS6+/+) mice subsequently demonstrated that RGS6 protein expression was upregulated in the ventral tegmental area (VTA) of the mesolimbic system following prolonged alcohol exposure. Conversely, studies performed in RGS6 knockout (RGS6^{−/−}) mice established that loss of RGS6 ameliorated not only alcohol seeking behavior but also those behaviors associated with alcohol-conditioned reward and withdrawal. Further inspection of the RGS6^{−/−} mice under control conditions revealed a reduction in the striatal DA suggesting that RGS6 might regulate DA production presynaptically, potentially through its ability to inhibit GPCR signaling. In support of this

hypothesis, daily intraperitoneal (i.p.) administration of a GABA_BR antagonist, SCH-50911, or a dopamine 2 receptor (D₂R) antagonist, raclopride, was associated with an increase in voluntary alcohol consumption in RGS6^{−/−} mice. Although it is not exactly clear how the GABA_BRs and D₂Rs regulate DA levels and thus alcohol seeking behavior, it has been hypothesized that they may do so by modulating the levels of the DA-synthesizing enzyme tyrosine hydroxylase (TH), the vesicular monoamine transporter 2 (VMAT2), and the dopamine transporter (DAT). Indeed, levels of TH and VMAT2 mRNA were lower in the VTA of RGS6^{−/−} animals compared to RGS6+/+ mice under basal conditions, and DAT mRNA levels were upregulated in RGS6^{−/−} mice following chronic alcohol exposure. Furthermore, i.p. injection of RGS6^{−/−} mice with the DAT inhibitor GBR-12909 promoted voluntary alcohol consumption in these mice to an even greater degree than either of the GABA_BR and D₂R inhibitors (21). These findings suggest that RGS6 inhibition of GPCR-mediated signaling may prevent upregulation of DAT and assure the normal synthesis and release of DA that is responsible for alcohol reward behaviors (Fig. 4a).

The evidence presented thus far indicates that RGS6 is critical for normal DA-mediated alcohol seeking behavior, thus identifying it as a viable therapeutic target. However, the advantages of RGS6 as a therapeutic target may not only reside in its ability to mediate alcohol seeking behavior but also in its ability to mediate signaling pathways that prevent alcohol-induced organ damage. In evidence of this fact, RGS6 deficiency was not only associated with blunted alcohol seeking behavior but also protection from the pathological effects of chronic alcohol consumption on peripheral tissues. In particular, RGS6^{−/−} mice chronically exposed to alcohol lacked alcohol-induced cardiac hypertrophy and fibrosis, hepatic steatosis, and gastrointestinal barrier dysfunction and endotoxemia. This reduction in alcohol-induced peripheral tissue damage is believed to involve RGS6's direct or indirect regulation of reactive oxygen species (ROS) production and the apoptotic cascade (21) similar to its functions in cancer suppression (27–30).

The results of this study, which describe RGS6 as a critical mediator of alcohol-associated reward behaviors, have established a foothold for RGS6 in the growing body of evidence which speaks to the importance of the R7 subfamily in modulating drug-induced reward behaviors and addiction. Indeed, both RGS7 and RGS9 have been strongly linked to these processes in models of morphine exposure and addiction (46–51). In the context of morphine addiction, which also involves modulation of neuronal signaling in the mesolimbic reward pathway, RGS7 and RGS9 appear to act primarily postsynaptically in neurons of the nucleus accumbens (NAc) to modulate the μ -opioid receptor (MOR), although they appear to have distinct functions (47, 48). Interestingly, there is also some preliminary evidence suggesting a potential role for the two remaining R7 family members, RGS6 and RGS11, in morphine responses (46, 51).

Anxiety and Depression

Deficits in serotonergic neurotransmission within the cortico-limbic-striatal neuronal circuit have been associated

Fig. 4. RGS6 in central nervous system diseases. Schematic outlining the role of RGS6 in alcoholism (a), anxiety and depression (b), and Parkinson's disease (c). Red indicates neurons projecting from the ventral tegmental area (VTA) in the meso-limbo-cortical pathway. Green indicates neurons projecting from the raphe nucleus (RN). Blue represents neurons projecting from the substantia nigra pars compacta (SNc) in the nigro-striatal pathway. **a** It is believed that RGS6 acts as a critical mediator of alcohol-seeking behaviors by inhibiting GABA_BR signaling which normally promotes upregulation of the dopamine transporter (DAT) and inhibits vesicular monoamine transporter 2 (VMAT2) and tyrosine hydroxylase (TH). Conversely, removal of RGS6, which is normally upregulated in the VTA following alcohol consumption, may ameliorate alcohol reward and withdrawal by promoting GABA_BR signaling decreasing dopamine (DA) bioavailability. **b** RGS6 promotes anxiety and depression by inhibiting the 5-HT_{1A} heteroreceptors in cortical and hippocampal neurons that synapse with serotonergic neurons of the RN. By blocking 5-HT_{1A} heteroreceptor-mediated inhibition of adenylyl cyclase (AC), RGS6 promotes the accumulation of cyclic AMP (cAMP) and the subsequent activation of protein kinase A (PKA) and cAMP responsive element-binding protein (CREB), all of which contribute to anxiety and depression and counteract the actions of antidepressant/anxiolytic medications. **c** RGS6 may also mediate the survival of dopaminergic SNc neurons by inhibiting dopamine receptor (D₂R) signaling in these neurons. By acting as a gatekeeper of D₂R signaling, RGS6 is believed to assure not only normal synaptic release of DA but may also prevent the accumulation of cytotoxic DA byproducts that could contribute to neuronal degeneration. PFC prefrontal cortex, STR striatum, NAc nucleus accumbens, HP hippocampus, DOPAL 3,4-dihydroxyphenylacetaldehyde, DOPAC 3,4-dihydroxyphenylacetic acid. Image adapted from references (21–23)

with both anxiety and depression. Many of the current therapies for the treatment of these disorders (e.g., selective serotonin reuptake inhibitors; SSRIs) seek to prolong serotonin (5-HT) synaptic presence and postsynaptic serotonergic signaling by inhibiting presynaptic 5-HT reuptake. However, the limited efficacy of these drugs, their off target effects, and the delay in their therapeutic onset (weeks to months) have promoted investigation into new treatment options.

Of particular interest, in the search for new antidepressants and anxiolytics were the 5-HT_{1A} receptors, which are GPCRs located in the cortical and hippocampal neurons that

are believed to mediate the antidepressant and anxiolytic effects of 5-HT (52–60). As such, it was hypothesized that regulation of these receptors might represent a new therapeutic strategy. This hypothesis was supported by the finding that mice expressing a knock-in mutation within Gα₁₂ (G148S), which disrupts RGS-mediated regulation of the 5-HT_{1A} receptor, not only have increased 5-HT_{1A} receptor signaling but also display spontaneous anxiolytic and antidepressant behaviors (61). However, while this study demonstrated that RGS modulation of 5-HT_{1A} receptor signaling is important for its antidepressant effects, it did not address which RGS protein was responsible for this regulation. RGS6 was later discovered

as a critical regulator of 5-HT_{1A} heteroreceptor signaling (22). Not only is RGS6 present in cortical and hippocampal neurons, as shown through immunohistochemistry and western blot, but RGS6^{-/-} mice also display spontaneous antidepressant and anxiolytic behaviors which are reversed through i.p. administration of WAY-100635, a 5-HT_{1A} receptor antagonist. Furthermore, RGS6 heterozygous (+/-) mice, which show similar levels of anxiety and depression as RGS6^{+/+} mice, are sensitized to the antidepressant effects of the SSRI, fluvoxamine, and the direct 5-HT_{1A} receptor agonist, 8-OH-DPAT (both administered i.p.). It is believed that the anxiolytic effects of RGS6 deletion are mediated through the potentiation of postsynaptic 5-HT_{1A} heteroreceptor-mediated inhibition of the adenylyl cyclase-cyclic AMP-protein kinase A-cAMP response element-binding protein (AC-cAMP-PKA-CREB) pathway. Evidence for the critical involvement of this pathway was demonstrated through a reduction in phospho-PKA and CREB activity in the cortex of RGS6^{-/-} mice. In addition, i.p. treatment of RGS6^{-/-} mice with the AC activator forskolin not only activated the AC-cAMP-PKA-CREB pathway in the cortex and hippocampus but also reversed the antidepressant phenotype associated with RGS6 deficiency (22). It should be noted at this point that there is a second population of 5-HT_{1A} receptors present on presynaptic serotonergic nerve terminals within the cortex and hippocampus, the 5-HT_{1A} autoreceptors. Activation of the 5-HT_{1A} autoreceptors reduces neuronal firing rate and inhibits the synthesis and release of 5-HT (62). However, RGS6 appears to primarily regulate the postsynaptic 5-HT_{1A} heteroreceptor as 8-OH-DPAT-induced hypothermia (dependent on the presynaptic 5-HT_{1A} autoreceptor (63)) was equally potent in RGS6^{+/-} and RGS6^{+/+} mice. As further evidence for the postsynaptic role of RGS6 in modulating 5-HT_{1A} heteroreceptor signaling, activation of the AC-cAMP-PKA-CREB pathway can be rescued in RGS6^{-/-} cortical neurons in culture directly through forskolin treatment (22) (Fig. 4b).

The study published by Stewart and colleagues (22), described above, establishes RGS6 as a critical mediator of anxiety and depression and compliments previous studies which have also linked other R7 family members, RGS7 and RGS9, to stress-related disorders. In particular, an intronic SNP in RGS7 (rs11805657) has been linked to panic disorder with comorbid agoraphobia (64). This is interesting as RGS7 is also expressed in the cortex and hippocampus like RGS6. However, RGS7 is likely acting through a signaling cascade that is separate from the 5-HT_{1A}R-AC axis as it did not appear to play a compensatory role in the absence of RGS6 (22), and RGS7 was not able to modulate 5-HT_{1A} receptor signaling *in vitro* (65). In addition, RGS7 is also upregulated in the locus coeruleus (LC) of the mouse following chronic stress induced by cold exposure and is responsible for modulating the ability of the α_2 -autoreceptor to inhibit neuronal firing and release of norepinephrine (66). Finally, there is evidence suggesting that RGS9 may play a modulatory role in anxiety as RGS9-2 expression is upregulated in the NAc in response to a mouse model neuropathic pain. Furthermore, RGS9-2^{-/-} mice show elevated levels of anxiety and depression after developing neuropathic pain symptomology (67).

Parkinson's Disease

Parkinson's disease is a progressive neurodegenerative disorder that is characterized by the death of dopaminergic neurons in the substantia nigra pars compacta (SNc) (68–70). These neurons normally project to the striatum where they help to regulate motor behavior. As such, loss of SNc dopaminergic neurons in Parkinson's disease is associated with bradykinesia, rigidity, and resting tremors. Despite knowing that degeneration of dopaminergic SNc neurons is responsible for Parkinson's disease, the molecular pathways underlying this degeneration are unknown, with less than 10% of Parkinson's cases displaying a clearly identified genetic component (71). The situation is further complicated by the fact that current mouse models developed to explore these known genetic components have not consistently replicated degeneration of dopaminergic SNc neurons (72).

Alternative avenues of investigation, utilizing rodent models with altered SNc neuron development, have recently opened new doors in Parkinson's research. In particular, characterization of mice deficient in the homeobox transcription factor pituitary homeobox 3 (PitX3) revealed that these mice not only show consistent loss of dopaminergic SNc neurons during mouse fetal development (73–75) but that these mice also have Parkinson's-like movement phenotypes that are partially reversed through levodopa (L-DOPA) treatment (76, 77). Further cementing the importance of PitX3 in Parkinson's research, genetic association studies identified PitX3 polymorphisms in non-familial cases of Parkinson's disease (78).

Despite the promising discoveries made in PitX3-deficient mice, it remained unclear why these mice suffered from developmental loss of dopaminergic SNc neurons. Therefore, a microarray analysis was conducted comparing gene expression in PitX3-dependent and PitX3-independent neuronal populations in the SNc. This analysis indicated that dopaminergic SNc neuron loss was strongly associated with the downregulation of RGS6 mRNA, identifying RGS6 as a potential survival factor (23). Indeed, it was further discovered using immunohistochemistry that the RGS6 protein is enriched in dopaminergic neurons of the SNc and that its expression was required for the survival of these neurons in adult animals. The importance of RGS6 for dopaminergic SNc neuron survival was evident in RGS6^{-/-} mice which suffered from age-onset neurodegeneration of these neurons by 1 year of age, degeneration which was not present in age-matched RGS6^{+/+} animals. SNc neurodegeneration in RGS6^{-/-} mice was correlated with markers of pathological change as well as a concomitant decrease in PitX3 and its target gene products: TH, aldehyde dehydrogenase 1 family, member A1 (Aldh1a1), brain-derived neurotrophic factor (Bdnf), and VMAT2 as measured by immunohistochemistry. Furthermore, several genes that had been previously associated with familial forms of Parkinson's such as DJ-1 (PARK7), Pink1 (PARK6), and Lrrk2 (PARK8) also showed altered protein expression in the degenerating SNc neurons of RGS6-deficient mice (23, 79).

Exactly how RGS6 mediates the survival of aging dopaminergic SNc neurons remains unclear. However, it is

likely that RGS6's role in SNc dopaminergic neuronal survival may be related to its ability to inhibit GPCRs. In evidence for this hypothesis, immunohistochemical analysis has revealed that there is an increase in phospho-Erk1/2 levels and an increase in glycosylated DAT expression in degenerating neurons (23). Changes in the expression of both of these proteins can be explained by an increase in D₂R signaling. Interestingly, expression of the D₂R was not increased in degenerating SNc neurons (23) and since it is a GPCR that signals *via* G $\alpha_{i/o}$, this leaves open the intriguing possibility that it is regulated by RGS6. Indeed, if the D₂R is regulated by RGS6, it would be predicted that its activity would be increased in the absence of RGS6, potentially resulting in not only increased DAT and phospho-Erk1/2 expression (as previously observed (23)) but also in the inhibition of both TH and VMAT2 (80, 81). TH and VMAT2 protein expression is likely also downregulated through the loss of PitX3 in RGS6-deficient SNc neurons, as mentioned earlier. In the end, RGS6 deficient neurons would be expected to not only have an impaired ability to synthesize (low TH) DA and package it into vesicles (low VMAT2) but would also increase their DA reuptake (high DAT). Together, these changes could increase cytosolic DA in SNc neurons causing neurodegeneration through accumulation of cytotoxic DA metabolites, such as 3,4-dihydroxyphenylacetaldehyde (DOPAL) (82, 83) (Fig. 4c).

The results of the study published by Bifsha and colleagues (23), described above, are important as they represent the first animal model of Parkinson's disease where loss of a single gene (RGS6) manifests as an age-onset form of the disease that closely resembles the human disease. However, RGS6 is not the only R7 family member that has been linked to the phenotypic manifestations of Parkinson's disease. There is also evidence suggesting that RGS9 expression may be upregulated in the striatum of patients with Parkinson's disease (84). In addition, research using the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesion model of Parkinson's disease in monkeys has demonstrated that viral overexpression of RGS9-2 in the striatum diminishes the development of L-DOPA-induced dyskinesia (LID) without minimizing L-DOPA's antiparkinsonian effects (85). Finally, RGS9^{-/-} mice that undergo the 6-hydroxydopamine lesion model of Parkinson's disease display an increased susceptibility to LID compared to their RGS9^{+/+} counterparts (85). These findings, however, implicate a postsynaptic role of RGS9 in the striatum *vs.* the proposed presynaptic role of RGS6 in dopaminergic neurons of the nigrostriatal pathway.

Alzheimer's Disease, Schizophrenia, and Eye-Related Disorders

So far, we have described current work elucidating the critical role of RGS6 in the disease pathology of alcohol addiction, anxiety/depression, and Parkinson's disease. However, this discussion likely only reflects the tip of the iceberg with regard to RGS6's role in CNS diseases. In fact, a new study detailing structural neuroimaging genetic interactions in Alzheimer's disease recently reported that a SNP in RGS6 (rs4899412) was significantly associated with volumetric changes in the caudate nucleus of Alzheimer's patients (24). Similarly, GWAS studies have also indicated that another

SNP in RGS6 (rs2332700) is significantly associated with schizophrenia (25). The possible significance of RGS6's role in schizophrenia is further supported by preliminary studies which also suggest that RGS7 and RGS9 may be differentially expressed in patients with schizophrenia (86, 87) and may modulate brain responses to psychostimulants and antipsychotics (49, 88–93). Finally, not only is there substantial evidence detailing the critical role of various R7 family members in proper vision (94–97) but a splice acceptor variant of RGS6 (c.1369-1G>C) has also been positively associated with the familial inheritance of congenital cataracts (26). Clearly, there is still significant work that needs to be done to elucidate the role of RGS6 in proper brain function.

RGS6 AND CANCER

GPCRs are overexpressed in numerous cancers and can drive tumor cell growth and metastasis. Consequently, GPCR signaling has become a point of interest in cancer biology (98). Given their ability to negatively regulate GPCR signaling, it is conceivable that RGS proteins might act as tumor suppressors or modulate carcinogenesis. In support of this hypothesis, RGS proteins were first linked to cancer in 2004, when it was discovered that a SNP in the *RGS6* gene (rs2074647) was positively associated with a reduced risk of bladder cancer, especially in smokers (27). The RGS6 SNP was found to increase translation/stability of RGS6 mRNA suggesting that an increase in RGS6 expression was responsible for its protective effect against bladder cancer, especially that induced by carcinogens. These findings provided the first glimpse into the critical role of RGS6 as a tumor suppressor and mediator of DNA damage signaling, which would be further demonstrated through subsequent studies. Of particular interest, it was found that these activities were due to G protein-independent actions of RGS6 (28).

Following the initial study demonstrating that a SNP in RGS6 was positively associated with a reduced risk of bladder cancer, there were three other studies that aided in cementing the role of RGS6 as a tumor suppressor. First, the same RGS6 SNP, identified in the previous bladder cancer study, was similarly linked to a reduced risk of lung cancer (99, 100). Second, and likely the best evidence for the role of RGS6 as a tumor suppressor, RGS6 expression was found to be negatively correlated with breast cancer progression in humans (28, 29). Furthermore, mice lacking RGS6 displayed both accelerated carcinogenesis in response to carcinogen exposure and developed spontaneous mammary tumors at an increased rate (further described below) (28). Finally, a similar trend has also been described in human pancreatic cancer, where RGS6 expression was once again found to be negatively correlated with tumor grade and prognosis (101). Together, these studies describe RGS6 as a potential tumor suppressor that is downregulated with tumor progression. To our knowledge, there is currently only one exception to these findings that has been described. In contrast to the studies described above, RGS6 mRNA is more highly expressed in ovarian cancer cell lines compared to non-cancerous IOSE cells. Here, RGS6 may act as a canonical RGS protein to

Fig. 5. Schematic outlining the link between RGS6 and various cancers. Previous research has shown that a SNP in human RGS6 increases RGS6 expression and is associated with a reduced risk of bladder and lung cancer. In support of a tumor suppressor role of RGS6, loss of RGS6 protein/mRNA expression is associated with gliomas, breast cancer, and pancreatic cancer in humans, and RGS6 suppresses breast carcinogenesis in mice. The precise role of the observed increase in RGS6 mRNA expression in various ovarian cancer cell lines is unclear, though RGS6 might function as a canonical G protein regulator to impair lysophosphatidic acid receptor 2 (LPA2)-mediated carcinogenesis. *Inhibitory signs* indicate possible tumor suppressor roles of RGS6. *Downward arrows* indicate reduced RGS6 expression

inhibit lysophosphatidic acid receptor 2 (LPA2) signaling, which drives progression of ovarian cancer (102). Figure 5 summarizes studies to date linking RGS6 to various cancers.

As was the case for the neurodegenerative diseases described above, RGS6 is not the only member of the R7 subfamily that has been shown to modulate cancer progression. Indeed, a SNP in RGS7 (rs6689169) has been linked to overall survival of patients with late-stage non-small cell lung cancer (99). In addition, RGS11 expression differs in oxaliplatin-sensitive vs. resistant colorectal cancer cell lines (103).

RGS6 Mediates Doxorubicin-Induced Cytotoxicity

Therapeutic strategies for breast cancer include surgery, hormonal therapies, radiotherapy, and adjuvant chemotherapies. Still, the treatment of breast cancer remains challenging in part due to the resistance that develops to radiation and conventional chemotherapeutic agents (104). Doxorubicin (Dox) is currently one of the most effective and widely employed chemotherapeutic agents and is used for the treatment of many types of cancer ranging from lymphoma, to breast cancer (105). Dox's therapeutic effects are mediated

via its ability to induce double strand DNA breaks (DSDBs) and activate the DNA damage response (DDR), by inhibiting topoisomerase II and promoting ROS generation (106–108).

Given the finding that a SNP in RGS6 can increase its expression and protect against smoking-related cancer, it was hypothesized that RGS6 may facilitate the DDR. Interestingly, experiments conducted to test this hypothesis not only confirmed that RGS6 facilitates DDR but that RGS6 is absolutely required for Dox-mediated activation of the ataxia telangiectasia mutant (ATM)-p53-apoptotic cascade in both mouse embryonic fibroblasts (MEFs) and the MCF-7 breast cancer cell line (30). First, it was found that Dox administration led to an upregulation in RGS6, which was accompanied by both the phosphorylation and upregulation of p53. Remarkably, the p53 response to Dox was almost completely absent in RGS6-deficient MEFs and MCF-7 cells, demonstrating that RGS6 is required for p53 activation. Second, it was found that RGS6 was also required for the autophosphorylation and activation of ATM, allowing the cell to sense/repair DSDBs or initiate the p53-dependent apoptotic cascade if DNA damage was too severe. Finally, transient expression of either RGS6 or its GAP-defective mutant was able to sensitize MCF-7 cells to a suboptimal dose of Dox, demonstrating that RGS6 promotes the activation of the ATM-p53-apoptosis pathway by G protein-independent mechanisms. In accordance with this latter finding, RGS6 was found to promote ATM activation by a recently identified oxidation mechanism (109). Indeed Dox-induced ROS generation was RGS6 dependent and both ATM activation and p53 phosphorylation were blocked with a ROS scavenger (30). Together, these findings revealed a novel mechanism for the therapeutic actions of Dox, namely, that RGS6 mediates Dox-induced, ROS-dependent activation of both ATM and p53. As such, RGS6 may represent a novel therapeutic target for the treatment of cancer (Fig. 6a).

RGS6 Functions as Tumor Suppressor in the Breast

Research describing how RGS6 was restrictively expressed in human breast ductal epithelial cells and lost in these cells with cancer progression (29) first suggested that RGS6 might function as a tumor suppressor. Of particular interest was the fact that RGS6 loss was universally correlated with increasing breast cancer tumor grade, independent of tumor status, *i.e.*, estrogen receptor (ER)/progesterone receptor (PR)/human epidermal growth factor receptor 2 (HER2) status (28). Therefore, to evaluate the potential role of RGS6 as a tumor suppressor, the effects of exogenous RGS6 expression on the proliferation of various cancer cell lines were explored. These experiments demonstrated that RGS6 possesses powerful antiproliferative and apoptotic activity in breast cancer cells (29). In terms of its antiproliferative effects, RGS6 suppressed growth by inducing G1/S phase cell cycle arrest and inhibited breast cancer colony formation. In addition, RGS6 was also able to induce the intrinsic apoptotic pathway in breast cancer cell lines, by promoting the generation of ROS. Interestingly, RGS6's ability to induce the intrinsic apoptotic pathway was independent of its GAP activity (29).

Fig. 6. Schematic illustrating the G protein-independent role of RGS6 in doxorubicin-induced apoptosis and antiproliferative signaling in the breast. **a** RGS6 is induced by doxorubicin (Dox) treatment and functions as a critical upstream mediator of reactive oxygen species (ROS)-dependent activation of the ataxia telangiectasia-mutated (ATM)-p53-apoptotic pathway in both mouse embryonic fibroblasts and breast cancer cells. Whether the RGS6-dependent upregulation of p53 in response to Dox induces autophagy by DNA-damage regulated autophagy modulator 1 (DRAM1) is unknown. **b** RGS6 is also a multifunctional tumor suppressor that simultaneously inhibits cell cycle progression and pro-growth signal downstream of human epidermal growth factor receptor 2 (HER2) and the estrogen receptor (ER). In addition, RGS6 functions as a scaffold protein to promote Tip60-mediated DNA (cytosine-5)-methyltransferase 1 (DNMT1) acetylation leading to its degradation and preventing DNMT1 silencing of pro-apoptotic genes. This represents an essential role of RGS6 in preventing reticular activating system (Ras)-induced oncogenesis. *R7BP* R7 family-binding protein, *DMAP1* DNA methyltransferase1 associated protein 1, *Met* methylation, *Ub* ubiquitin, *Ac* acetylation, *ERE* estrogen response element. Image adapted from references (28, 30, 110)

To determine whether RGS6 functions as a bona fide tumor suppressor in the breast, the effects of RGS6 loss on spontaneous and DMBA (7,12-dimethylbenza (α) anthracene)-induced breast carcinogenesis were compared in mice (28). As in human breast specimens, RGS6 (but no other R7 family members) was found to be restrictively expressed in the ductal epithelial cells of RGS6^{+/+} mammary glands and was downregulated upon DMBA treatment. Furthermore, while DMBA treatment induced tumor formation in both RGS6^{+/+} and RGS6^{-/-} mice, DMBA-induced mammary tumor initiation and growth was accelerated in RGS6^{-/-} compared to RGS6^{+/+} mice, resulting in a reduced survival. In further support of the increased sensitivity of RGS6^{-/-} mice to tumor formation, it was found that 20% of aged virgin female RGS6^{-/-} mice developed spontaneous tumors compared to 0% of their RGS6^{+/+} cohorts. In an effort to account for this increased sensitivity of RGS6^{-/-} mice to tumorigenesis, mammary glands and mammary epithelial cells were isolated from RGS6^{-/-} and RGS6^{+/+} mice and examined for differences in markers of apoptosis and oncogenesis. These studies revealed

that DMBA-induced activation of the ATM-p53-apoptotic pathway was significantly reduced in ductal epithelium of RGS6^{-/-} mice compared to RGS6^{+/+} controls. In addition, RGS6^{-/-} mice showed greater DMBA-induced increases in Cyclin D1 and DNA (cytosine-5)-methyltransferase 1 (DNMT1) expression in the ductal epithelium compared to RGS6^{+/+} controls. Further experiments demonstrated that RGS6^{-/-} mammary epithelial cells (MECs) exhibited increased basal levels of heregulin- and estradiol-stimulated proliferation compared to RGS6^{+/+} controls. Finally, DMBA-induced ROS generation and activation of the ATM-p53-apoptotic pathway were reduced in RGS6^{-/-} compared to RGS6^{+/+} controls. Together, these experiments demonstrate that RGS6 has a dual role in suppressing tumor formation by curbing cellular proliferation and by facilitating initiation of the ATM-p53-apoptotic cascade (28).

Based upon the findings of Maity and colleagues (28), described above, it has been proposed that RGS6 is a previously unrecognized tumor suppressor in the breast. RGS6's robust expression in ductal epithelial cells, which

undergo malignant transformation, may serve a crucial role in defending against oncogenic or genotoxic stress. Figure 6b details the current model of RGS6 tumor suppression, in which RGS6 blocks cellular transformation and tumorigenesis by facilitating activation of the DDR and apoptosis, effectively halting HER2-, ER- and carcinogen-induced cellular proliferation. The universal loss of RGS6 in breast cancers, independent of their molecular classification, suggests that RGS6 stands as a major barrier to tumor initiation and progression irrespective of the oncogenic stimulus (Fig 6).

RGS6 Inhibits Ras-Induced Cellular Transformation

The reticular activating system (Ras) proto-oncogene is critical for the proper regulation of cell proliferation. As such, point mutations leading to oncogenic activation of Ras have been found in a large number of human cancers. DNMT1 is overexpressed in many cancers as well (111–121), as it is required for the silencing of tumor suppressor genes essential for Ras-induced oncogenic cellular transformation (122–124). The canonical functions of DNMT1 include maintenance of genomic DNA methylation patterns in proliferating cells and methylation of CpG islands in promoter regions, a key mechanism for silencing gene expression (125, 126). As mentioned earlier, it is clear that DNMT1-dependent, DNA methylation-mediated silencing of tumor suppressor genes is essential for tumor development and progression as well as transformation by oncogenes, such as Ras. Therefore, it was proposed that a link might exist between RGS6 and DNMT1, a hypothesis supported by the fact that RGS6 forms a complex with DNMT1 through its binding with DNMT1-associated protein 1 (DAP1) (127). A possible functional link between RGS6 and DNMT1 was further suggested as DMBA treatment induced an increase in DNMT1 expression in ductal epithelium of DMBA-treated RGS6^{-/-} mice compared to RGS6^{+/+} controls (28) (Fig. 6b).

Further studies demonstrated that RGS6 is not only a tumor suppressor itself but is also induced by oncogenic Ras and blocks Ras-induced cellular transformation through a novel DNMT1-dependent mechanism (110). In this study, initial experiments confirmed that cellular transformation induced by oncogenic Ras and dominant negative p53 was increased in RGS6^{-/-} MEFs compared to RGS6^{+/+} MEFs. Immunoblots revealed that Ras was able to induce RGS6 and DNMT1 expression in RGS6^{+/+} MEFs and that RGS6^{-/-} MEFs exhibited a significant increase in basal and Ras-induced DNMT1 expression. Further experiments demonstrated that the application of a DNMT1 inhibitor prevented Ras-induced cellular transformation in RGS6^{-/-} MEFs, indicating that RGS6 suppressed Ras-induced transformation through the upregulation of DNMT1. Indeed, RGS6^{-/-} MEFs exhibited a loss of DNMT1 pro-apoptotic gene expression so that the expression of these genes was equivalent to that observed in Ras-transformed RGS6^{+/+} MEFs. These experiments thus identified a critical role of RGS6 in regulating DNMT1 expression and preventing its oncogenic actions. Subsequent experiments in this study showed that RGS6 promotes DNMT1 degradation by scaffolding DNMT1 and the

acetyltransferase Tip60 to facilitate DNMT1 acetylation which is followed by its subsequent ubiquitylation and degradation (110) (Fig 6b).

The experiments described above (110) provided evidence for a novel and crucial role for RGS6 in the suppression of oncogenic transformation. This work also provided new insights into the mechanisms responsible for regulating DNMT1 expression and activity. In addition, these studies revealed that Tip60 associates with RGS6 *via* its RGS domain, providing the first evidence for a novel function of the RGS domain beyond G protein regulation. Thus, RGS6 loss may be responsible for the upregulation of DNMT1 and the increase in DNA methylation associated with carcinogenesis. Importantly, the ability of RGS6 to inhibit oncogenic transformation by promoting DNMT1 degradation once again identifies RGS6 as potential target for treatment of human cancers.

CONCLUSION

The studies described here suggest that RGS6 is a critical modulator of both G protein-dependent neurotransmission, whose alteration is associated with several CNS pathologies, and G protein-independent pro-apoptotic and growth suppressive mechanisms, associated with cancer pathology and Dox resistance. Together, these findings implicate RGS6 as a novel therapeutic target for the treatment of cancer and CNS diseases. Therefore, future research should focus on identifying compounds that activate or inhibit RGS6. Such research will likely be aided by determining the functions of the various RGS6 alternative splice forms present in both CNS and peripheral tissues.

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