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RGS17: an emerging therapeutic target for lung and prostate cancers

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Abstract

Ligands for G-protein-coupled receptors (GPCRs) represent approximately 50% of currently marketed drugs. RGS proteins modulate heterotrimeric G proteins and, thus, GPCR signaling, by accelerating the intrinsic GTPase activity of the G α subunit. Given the prevalence of GPCR targeted therapeutics and the role RGS proteins play in G protein signaling, some RGS proteins are emerging as targets in their own right. One such RGS protein is RGS17. Increased RGS17 expression in some prostate and lung cancers has been demonstrated to support cancer progression, while reduced expression of RGS17 can lead to development of chemotherapeutic resistance in ovarian cancer. High-throughput screening is a powerful tool for lead compound identification, and utilization of high-throughput technologies has led to the discovery of several RGS inhibitors, thus far. As screening technologies advance, the identification of novel lead compounds the subsequent development of targeted therapeutics appears promising.

Cells maintain homeostasis and communicate with their surrounding environment. This occurs through complex signaling pathways involving, in part, chemical modification of proteins as well as specific protein-protein interactions (PPIs). These signaling cascades are often initiated through the activation of membrane-bound receptors. One of the most prevalent of these cellular receptors are the G-protein-coupled receptors (GPCRs). GPCRs are typified by seven transmembrane helices, an N-terminus extending into the extracellular environment and a C-terminus in the intracellular milieu. Members of this family include receptors for many hormones, neurotransmitters, chemokines and calcium ions [1]. Since their discovery, GPCRs have become the target for approximately 50% of all currently marketed therapeutics [2]. GPCRs couple to heterotrimeric G proteins that consist of a G α subunit and a $\beta\gamma$ heterodimer [3]. The G α subunit of the G protein binds GDP in the basal, inactive state. Activation of the GPCR by an extracellular ligand initiates a series of coupled

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conformational changes, first in the GPCR and then the $G\alpha$ subunit, releasing GDP and allowing GTP to bind – causing the $G\alpha$ and $\beta\gamma$ heterodimer to disassociate and signal through their respective intracellular effectors [4]. Hydrolysis of the bound GTP to GDP by the intrinsic GTPase activity of the $G\alpha$ subunit induces the re-association of α and $\beta\gamma$ subunits, terminating the signaling cascade [5].

RGS proteins temporally regulate a subset of these signaling events, serving as GTPase accelerating proteins (GAPs) for inhibitory $G\alpha$ -coupled GPCRs by increasing the intrinsic rate of hydrolysis of G proteins and acting to terminate GPCR signaling much more rapidly. RGS proteins are a diverse protein family with unique tissue distributions for each member and are strongly related by signal transduction events [6]. The first of this unique family of proteins was discovered in yeast genetic studies of GPCR signaling and later members by using sequence homology within a conserved 120-amino acid domain, known as the RGS homology (RH) domain or ‘RGS box’ identified from the founding member [7–10]. Since these early discoveries, approximately 30 members of the RGS superfamily have been characterized. Many RGS proteins contain multiple structural and functional motifs, and domains by which they mediate cross-talk between GPCR-dependent and -independent signaling pathways [11]. The presence of these specific accessory domains further divides RGS proteins into subfamilies (Figure 1).

Studies of the GPCR signaling pathways have implicated these signaling cascades in multiple disease states that range from cancer and heart disease to immunological and neurological pathologies [11–17]. Furthermore, the successful targeting of the GPCRs is still just a small fraction of the possible therapeutic potential of the GPCR pathway. Because of their ability to regulate multiple signaling events in a temporally and tissue-specific manner, RGS proteins present a unique opportunity for drug discovery. Targeting RGS PPIs that govern select signals downstream of GPCRs is challenging, but expands the number of potential targets and allows exploitation of the discrete roles that specific interactions may play within cellular signaling cascades [5].

The RGS subfamilies

Since their discovery in the early 1990s, the RGS superfamily of proteins has grown to contain eight subfamilies. These subfamilies all contain the RH domain but are classified based on a diverse array of accessory domains. The classical RGS proteins fall into one of four groups: A/RZ; B/R4; C/R7; and D/R12. These four subfamilies differ drastically in number of accessory domains present, while still maintaining GAP-competent RGS boxes. The B (or R4) family is the largest; this group consists of nine RGS proteins that feature only the RH domain with an N-terminal amphipathic α -helical region [18]. The C (or R7) subfamily has four members that are larger due to the presence of N-terminal disheveled/Egl-10/pleckstrin and G-protein γ subunit-like domains [19–23]. These two accessory domains contribute to membrane localization by binding R7-binding proteins and stability through binding the irregular $G\beta$ protein, $G\beta 5$, respectively [19–22,24]. The D (or R12) subfamily of RGS proteins is home to the largest classical RGS proteins discovered to date. These large proteins contain multiple accessory domains such as the PSD-95/Dlg/ZO-1, phosphotyrosine-binding, Ras-binding and the $G\alpha_{i/o}$ -Loco interaction domains [25–27]. Each of these domains contributes to the multiple functions and downstream signaling interactions of the R12 subfamily of RGS proteins. The A (or RZ) subfamily, made up of four RGS proteins, are classified by an N-terminal cysteine-rich cluster or ‘cysteine string’ [18]. This motif is believed to be heavily palmitoylated, leading to tight membrane association [28].

The founding member of the RZ subfamily is GAIP, now known as RGS19. RGS19 was the first mammalian RGS protein identified via the yeast two-hybrid system, with $G\alpha_{i3}$ serving as 'bait' [29]. Additional members of this family (RGS17/RGSZ2 and RGS20/RGSZ1) were isolated by yeast two-hybrid screens using $G\alpha_z$ and $G\alpha_o$ as bait [30,31]. RGSZ2 (RGS17) was discovered using $G\alpha_o$ as bait in a screen of chick dorsal root ganglion neuron cDNA [31]. RGS20 was also identified by purification and protein sequencing of a bovine brain $G\alpha_z$ -specific GAP activity [30]. The last member of the RZ family is Ret-RGS, which is a splice variant of RGS20 and was originally discovered as a GAP for transducin ($G\alpha_t$) in the retina [32]. The focus of this review is RGS17, a member of the RZ family, and its potential as a therapeutic target in multiple disease states.

RGS17 is conserved between species, the human protein has >90% similarity to rat, chicken and mouse [33,34]. This similarity increases when comparing the RH domain of RGS17 with other RZ members [33,34]. More recently, it has been hypothesized that the cysteine string undergoes multiple post-translational modifications to regulate subcellular localization. Future work will shed light on the regulation of expression of RGS17, degradation and subcellular localization.

Expression & function of RGS17

With approximately 30 members in the RGS family, determining specificity of different RGS proteins for each $G\alpha$ subunit as well as determining tissue distribution has been essential. This is especially true when one considers RGS proteins as a potential therapeutic target, as off-target effects limit the utility of drugs significantly. When considering RGS17, tissue distribution was first reported in 2001 with the identification of RGS17 (RGSZ2) in rat atrial myocytes [35]. RGS17 was found to be expressed in 85–90% of the 12 atrial myocytes tested, and was one of only seven of the 20 RGS proteins tested to show expression in the atrial myocytes.

Selective RGS expression was further characterized in 2003 with an expansive study of 19 RGS proteins and their respective expression levels in an array of organs and tissues by the Garnier group [36]. In this examination, RGS17 distributed almost exclusively to the brain. Upon subsequent examination by PCR, RGS17 mRNA levels were expressed at higher levels in the cerebellum, nucleus accumbens, parahippocampal gyrus and the putamen than other regions of the brain [37].

As highlighted above, RGS17 is a member of the RZ family of RGS proteins, characterized by the RGS domain and a cysteine rich N terminus, as well as their ability to regulate $G\alpha_z$ signaling pathways. RGS17 also regulates $G\alpha_{i/o}$ and $G\alpha_q$ signaling pathways [34]. RGS17 was identified as a regulator of $G\alpha_o$ signaling in a yeast two-hybrid screen of human brain cDNA libraries using constitutively active $G\alpha_o$ as bait. Selectivity was established when RGSZ1, the RZ family member most homologous to RGS17, was also tested against $G\alpha_o$ and was found to have minimal effect. Given its novel interaction with human $G\alpha_o$, RGS17 was also tested against other $G\alpha$ subunits and was found to regulate $G\alpha_i$ and $G\alpha_q$ signaling, but had no activity against $G\alpha_s$ [35].

RGS17: traditional signaling functions

RGS17 in μ -opioid receptor signaling

Previous studies have shown that desensitization of μ -opioid receptors in the CNS was mediated more effectively by $G\alpha_z$ than by $G\alpha_i$. In addition, members of the RZ subfamily (RGSZ1 and GAIP) were demonstrated to regulate $G\alpha_z$ proteins following activation by μ -opioid receptors, with GAIP regulating $G\alpha_z$ to a lesser extent [37]. This led to investigation

of another RZ subfamily member, RGS17, and the potential role it plays in μ -opioid receptor signaling. RGS17 was found to play a significant role in signal regulation of the μ -opioid receptor. RGS17 downregulation by antisense oligodeoxynucleotide injection into the lateral ventricle of mice showed an increase in potency and duration of morphine-induced antinociception at low doses. Higher doses of morphine resulted in an elongated desensitization of the receptor [38]. G-protein α subunits $G\alpha_o$, $G\alpha_i$ and $G\alpha_z$ were found to co-precipitate with the μ -opioid receptor, an effect that was reduced upon treatment with 10 nmol of morphine. In addition, both RGS17 and RGSZ1 were observed to co-precipitate with the μ -opioid receptor, although RGS17 sets itself apart from its RZ subfamily counterpart in that only RGS17 was reported to increase co-precipitation with $G\alpha$ subunits in response to a dose of 10 nmol morphine. This was most profound in $G\alpha_z$:RGS17 co-precipitation, which was increased as much as 70% over control cells [38].

Further studies demonstrated that post-translation modifications of RGS17 both act as scaffolds for its function and regulate the activity of RGS17 in μ -opioid receptor signaling [39,40]. Investigations into different isoforms of RGS17 led to the identification of two sumoylation motifs in RGS17 [39]. This would later be expanded to show that RGS17 contains two small ubiquitin-like modifiers- (SUMOs) interacting motifs and one sumoylation consensus site [40]. Immunoprecipitation studies showed that both RGS17 and RGSZ1 conjugate to SUMO-1, -2 and -3 but not to ubiquitin. Further studies showed that it is these sumoylated forms of RGS17 and RGSZ1 that are interacting with the μ -opioid receptor and corresponding $G\alpha$ subunits. Given the role RGS17 was shown to play in morphine-related μ -opioid receptor signaling, the effect of morphine administration on sumoylation was assessed. It was found that administration of morphine does not alter the degree of sumoylation of RGS17 [39].

Interestingly, Garzón and co-workers found that sumoylation does not alter the affinity of RGS17 for $G\alpha_i$, but it does affect GAP activity. Recombinant sumoylated RGS17 was demonstrated to have little or no GAP activity to $G\alpha_i$ in a single turnover assay. RZ family members have the unique ability to bind with comparable affinities to both GTP-bound $G\alpha$ and transition state $G\alpha$ subunits, and sumoylation did not affect the affinity of RGS17 for the transition state, but did delay the release of GDP. Sumoylation was determined to be a key factor in RGS17 localization to the plasma membrane [40].

As with many biological signaling pathways, the μ -opioid receptor has overlapping signaling with other pathways. The role of RGS17 in those overlapping pathways, specifically the μ -opioid receptor-mediated potentiation of *N*-methyl-D-aspartate receptor-calmodulin-dependent protein kinase II pathway via neural **nitric oxide synthase** regulation, has been investigated [41]. RGS17 was found to be essential for neural nitric oxide synthase (nNOS) recruitment to the μ -opioid receptor upon receptor activation, that is, the binding of morphine. Biomolecular fluorescence complementation assays demonstrated that this RGS17–nNOS interaction was through a PDZ-binding motif found on RGS17, which binds to a PDZ domain located on the N-terminus of nNOS. This PPI was found to impair nNOS activity, pointing to RGS17 having a role in nNOS regulation and subsequent nitric oxide production, resulting in the release of zinc from intracellular stores in response to morphine binding. This nNOS regulatory mechanism is attenuated upon RGS17 binding to $G\alpha$ subunits [41]. As stated above, RGS17 downregulation results in increased potency of morphine at low doses and an elongated desensitization at high doses [38]. Inhibition of nNOS and other PKC/NMDAR/CaMKII pathway proteins restores normal μ -opioid receptor function when RGS17 is downregulated [41].

Given the novel function of RGS17 as a regulator of zinc release, it was determined that RGS17 had direct interaction in the release of zinc [42]. Exposure of the cysteine-rich

domain of RGS17 to nitric oxide donors resulted in the release of zinc. Examination of the N-terminus of RGS17 revealed two zinc-binding motifs that coordinate the binding of zinc to four cysteine residues in a tetrahedral conformation. In addition, RGS17 was found to be essential for the regulation of intracellular zinc stores, as RGS17 knockdown mice exhibited the release of lower levels of endogenous zinc, ranging from non-detectable to moderate [42]. Zinc was observed to support the translocation of several downstream signaling proteins to the GPCR environment, where RGS17 directly interacts with the GPCRs, as shown by biomolecular fluorescence complementation assays. This differs from RGS17 interaction with the μ -opioid receptor, which requires the histidine triad nucleotide-binding protein 1 for recruitment [41]. RGS17 interactions as a regulator of zinc stores identifies it as a potential redox transducer, which is itself regulated by GPCRs [42].

Considering the establishment of RGS17 as a zinc regulator, and with the identification of SUMO-interacting motifs on RGS17, it is entirely possible that RGS17 plays a yet unidentified role in other regulatory processes in the cell. The presence of alternate binding domains in the various families of RGS proteins allows for a multitude of functionalities, in addition to the GAP function shared by all RGS members. Several RGS proteins have been identified as localizing in both the cytoplasm and nucleus, while others have already been shown to have activity in the nucleus [43–49]. In addition, sumoylation has been identified in a variety of cell events, both cytoplasmic and nuclear, including transport between the cytoplasm and nucleus, gene transcription and ion transport [50]. Thus, it is entirely plausible to speculate that RGS17 could exhibit additional cellular functions, including being directly responsible for the suppression of tumor-suppressing genes *FoxO4* and *Hnt*, although additional studies must be completed before a definitive interaction can be confirmed.

RGS17 expression & distribution changes in dopamine pathways

As discussed above, RGS17 is highly expressed in areas of the CNS [36]. Its roles in signaling pathways in these areas are still being studied. Dopamine receptors, all of which are GPCRs, are implicated in various clinical conditions, including the mediation of behavioral responses to drugs of abuse such as cocaine and amphetamines, which has been attributed to dopamine receptor D₁ (D₁R) [51]. Prenatal exposure to cocaine has been demonstrated to cause a permanent reduction in D₁R signaling in a rabbit model [52]. Microarray and *in situ* hybridization studies of D₁R knockout mice striatum, medial frontal cortex and visual cortex, showed region-selective reduction in *RGS2*, *RGS4*, *RGS9* and *RGS17*, with *RGS17* expression especially decreased in the medial frontal cortex [53]. Rabbit model prenatal exposure to cocaine was expected to show the same expression patterns as the D₁R knockout mice since prenatal exposure results in a permanent reduction in D₁R signaling [45]. However, results demonstrated an increase in *RGS17* expression levels in rabbit prenatal cocaine exposure [46]. The effect of D₁R receptor knockout on RGS protein expression levels is interest, in that RGS proteins are not known to bind to stimulatory G proteins that couple to D₁R.

Alterations in D₂-like receptor (dopamine receptors D₂, D₃ and D₄) expression and sensitivity have also been reported to be linked to clinical conditions such as schizophrenia, ADHD and bipolar disorder [54]. D₂-like receptor sensitivity can be achieved in rat model by administering quinpirole to rats during development that persist through the animals lifetime, a technique known as D₂ receptor priming [55,56]. *In situ* hybridization studies of these D₂ primed rats demonstrated that *RGS17* levels were increased in the striatum, nucleus accumbens and frontal cortex while *RGS9* expression levels were decreased in these areas [54]. The mechanism resulting in these changes in *RGS17* expression, both in regards to D₁ and D₂ signaling, have yet to be elucidated. Both could be a result of interaction cascades

that extend to the μ -opioid receptor systems. For example, in examining *RGS17*s increased expression upon D₂ priming, quinpirole has been shown to reduce the discriminative effects of μ -opioid agonists [57]. Alternatively, the increase in *RGS17* expression due to D₂ priming may be compensatory for the reduced expression of *RGS9* [54].

RGS17 expression influence on substance dependence

Studies have shown correlations between variations of the μ -opioid receptor gene *OPRM1* and κ -opioid receptor gene *OPRK1*, and alcohol and drug dependence [58–64]. The *OPRM1* and *OPRK1* genes have been shown to be linked to the *RGS17* and *RGS20* genes, respectively [65]. As stated above, both are members of the RZ subfamily and have been reported to associate with the μ -opioid receptor [38]. Given this relationship, Zhang and co-workers examined whether *RGS17* or *RGS20* polymorphisms might modulate substance dependence traits. *RGS20* did not demonstrate any potential modulation effects. Genetic association studies showed that seven of 13 identified *RGS17* single nucleotide polymorphisms (SNPs) to be associated with substance-dependence traits. Six were shown to be associated with one or more of the substance dependence traits examined (alcohol, cocaine, opioid and marijuana dependencies) in both populations examined (African American or European American). The remaining SNP (rs596359) was found to be associated with all of the dependencies in both populations [66]. This SNP was found much more frequently in the substance dependence cases than the control cases. It was found to be located in the *RGS17* promoter region, suggesting that the mechanism of increased risk of substance dependence may be by influencing *RGS17* transcription, although further studies will be required to fully elucidate the mechanism [66].

Variation of RGS17 expression in cancer & chemoresistance mechanisms

In the USA, lung cancer is the leading cause of cancer related deaths for both men and women, and is associated with a poor prognosis of a 15% survival rate over 5 years [67]. While smoking is still the major factor for increased risk of lung cancer, susceptibility is also attributed to genetic factors [68]. Genome wide linkage studies conducted by the Genetic Epidemiology of Lung Cancer Consortium identified a major susceptibility locus on chromosome 6q23–25 [69]. Fine mapping of this chromosome resulted in the identification of SNPs that associated to 6q23–25 in cases of familial lung cancer. Three of these were chosen upon the basis of having the strongest association to 6q23–25. All three were found to reside in a linkage disequilibrium block of 43 kilobases within the first intron of the *RGS17* gene [70].

Fine mapping studies did not find these specific SNPs in cases of sporadic lung cancer, however real-time qualitative PCR (RT-QPCR) studies using patient matched samples showed that 77% of sporadic tumor samples had increased expression of *RGS17* by an average of 9.1-fold. The initial RT-QPCR study examined only 13 tumor samples, but subsequent research expanded the sample size to 61 patient matched samples, and further solidified the relationship with *RGS17* being overexpressed in 80% of samples tested by an average of 8.3-fold [70].

To investigate the causal nature of this relationship, RNA (shRNA)-mediated knockdown of *RGS17* in H1299 non-small-cell lung cancer cells was performed. The knockdown cells exhibited a decreased proliferation rate and reduced colony formation. Subsequent injection of the knockdown and control H1299 cells in to nude mice revealed that over a period of 4 weeks the *RGS17* knockdown resulted in a tumor volume and mass decrease of 385 mm³ to 47 mm³, and 148 mg to 23 mg, respectively [70].

Microarray data revealed an increase of *RGS17* expression in prostate cancer cells compared with noncancerous prostate cells. RT-QPCR analysis of a small subset of five patient matched prostate cancer samples reported an increase in *RGS17* expression, which was detected in all prostate cancer tissue samples by an average of 7.5-fold [71]. shRNA knockdown of *RGS17* was performed in H1299, Hct116 and DU145 cell lines (lung cancer, colon cancer and prostate cancer cell lines, respectively), which resulted in decreased proliferation. The decreased proliferation was generally correlated to the degree of *RGS17* knockdown as determined by RT-QPCR [71]. Stable transfection of *RGS17* in to H1299 cells to cause *RGS17* overexpression resulted in the opposite effect, with *RGS17* overexpression causing an increase in cell proliferation. This effect was not found to be as robust as the *RGS17* knockdown, potentially due to the relatively high expression of *RGS17* in these cells [71].

In an effort to elucidate the mechanism by which *RGS17* regulates cell proliferation in tumor cells, microarray studies to identify genes whose expression was modified in response to changes in *RGS17* expression were carried out by James and colleagues. Two genes, *FoxO4* and *Hnt* were identified as being upregulated in response to *RGS17* knockdown. General regression tests of six published microarray data sets identified a list of genes that are co-expressed with *RGS17* in lung tumors. A large portion of these (56.3%) were genes that function within the CNS, as *RGS17* does. A smaller subset (32%) were found to be putative cAMP-response element binding (CREB) protein response genes [71].

RGS17 knockdown cells demonstrated both a decreased basal expression of CREB responsive elements as well as a decreased ability of forskolin to induce expression of CREB-responsive elements. This indicates that *RGS17* plays a positive modulatory role in the PKA–CREB pathway, consistent with known function in attenuating $G\alpha_{i/o}$ signaling. Forskolin treatment of control cells also induces proliferative activity in H1299 cells, an effect that is not significantly induced in *RGS17* knockdown H1299 cells. Supporting that *RGS17* is a factor in PKA–CREB modulation, H1299 control cells were treated with H89, a PKA inhibitor, which resulted in decreased proliferative activity. Subsequent overexpression of *RGS17* rescued the proliferative activity of the control cells [71].

As stated above, a major susceptibility locus for familial lung cancer was identified on chromosome 6q23–25, which contained three SNPs that were associated with the first intron of *RGS17* [70]. Other chromosomal loci have been identified as susceptibility markers for familial lung cancer, including variants found on chromosome 15q24–25.1, 5p15.33 and 6p21.33 [72–77]. These multiple susceptibility loci were investigated for cumulative effect. The individual risk factors as a percentage of total risk were determined for these four loci and the population attributable risk (PAR) was determined for 15q24–25.2, 5p15.33 and 6p21.33, as well as for all four loci combined. The results were an 8.5% risk factor for 15q24–25.1, 7.5% for 5p15.33 and 2.7% for 6p21.33, which resulted in a PAR of 17.6% for familial lung cancer. The 6q23–25/*RGS17* susceptibility locus resulted in an additional 20.6% PAR increase, and the cumulative PAR of all four susceptibility loci resulted in a PAR of 34.6%. In more general terms of increased susceptibility, subjects who had at least one copy of risk allele at each of the four regions had a resultant three- to 11-fold increased risk of familial lung cancer [78].

RGS17 was recently verified in a genome-wide association study as being a susceptibility marker for prostate cancer. The association of *RGS17* with susceptibility was determined to be very significant, exhibiting the lowest p value at 4.34×10^{-18} , a full two orders of magnitude lower than the next lowest p value [79].

RGS17 is upregulated in lung and prostate tumors, and plays a role in modulation of tumor cell proliferation through the PKA–CREB pathway [71]. In short, the $G\alpha_{i/o}$ and $G\alpha_z$ subunits inhibit adenylate cyclase and downregulate cAMP formation, which attenuates the cAMP–PKA–CREB signaling cascade. In response to the elevated cAMP levels in the cell the C-PKA subunits move into the nucleus where they phosphorylate the transcription factor, CREB [80–82]. Recently, it has been reported that CREB is involved in the development and tumorigenesis of endocrine tissues and lung adenocarcinoma [83,84]. Normal levels of RGS17 serve to modulate the cAMP–PKA–CREB signaling cascade, however in lung cancers *RGS17* transcripts are increased in 80% of tumors by an average of 8.3-fold over patient-matched normal lung tissues. The increase in RGS17 acts to attenuate $G\alpha_{i/o}$ and $G\alpha_z$ signaling, and results in increased cAMP in tumor cells, which activates the cAMP–PKA–CREB pathway that induces tumor cell proliferation and tumorigenesis [70,71]. The exact mechanism by which RGS17 is regulated in tumor progression has yet been unexplored.

Sun and colleagues investigated the role of miRNAs in the regulation of RGS17 in lung and prostate tumors [85]. Using binding software, hsa-mir-182 was identified as a mRNA that consistently and most efficaciously bound to the 3' UTR of *RGS17* with the strongest inhibitory effect. Three hsa-mir-182 binding sites were located in this region. Mutation experiments revealed that a single mutation in two of the three sites resulted in loss of hsa-mir-182 RGS17 suppression activity [85].

To determine if the identified miRNA had *in vivo* effect, hsa-mir-182 was virally infected in to CRL-5803 lung cancer cell lines. RTQPCR of the infected cell lines demonstrated a decreased *RGS17* expression compared with the control cells [85]. As previously reported, RGS17 knockdown significantly reduces tumor cell line proliferation [70,71]. Infection of hsa-mir-182 into two separate non-small-cell lung cancer cell lines (CRL-5803 and CRL-5889) mimicked the effects of RGS17 knockdown, resulting in decreased cell proliferation in the infected cells. This effect was attenuated upon subsequent transfection of *RGS17* [85]. In a panel of ten pairs of samples (cancer and control tissues per pair), five out of ten demonstrated a negative relationship between *RGS17* and hsa-mir-182 expression, although the results were not statistically significant [85].

RGS17 upregulation is not the only mechanism by which RGS17 can affect cancer. RGS17 downregulation has been shown to be important in developing chemoresistance in ovarian cancer [86]. Lysophosphatidic acid (LPA) has previously been identified as a mediator of ovarian cancer cell growth and survival [87,88]. LPA functions through activation of a family of GPCRs, which are regulated by RGS proteins. Three separate chemoresistant ovarian cancer cell lines were found to have significant decreases in the expressions of *RGS2*, *RGS10* and *RGS17*. *RGS5* was also found to exhibit decreased expression levels in two of the three of these chemoresistant cell lines [86]. Hooks and co-workers reported that SKOV-3 ovarian cancer cells treated for 24- and 48-h with cisplatin resulted in a decrease in *RGS10* and *RGS17* expression levels that persisted for more than 48 h after removal of the drug [86]. Subsequent siRNA knockdown of RGS10 and RGS17 in the absence of cisplatin treatment demonstrated increased cell viability over control cells over a period of 72 h. RGS10 or RGS17 siRNA knockdown in cell lines illustrated significantly higher percentage cell viability than controls when treated with cisplatin, vincristine and docetaxel. Conversely, RGS10 and RGS17 overexpression in SKOV-3 cells caused an observed increase in sensitivity to the chemotherapeutics [86]. The same study reported that overexpression of either RGS17 or RGS10 resulted in a decreased ability of LPA to stimulate AKT phosphorylation and activation and, thus, promote ovarian cancer cell survival [86].

Downregulation of RGS17 does not appear to be the only mechanism by which RGS17 promotes cancer resistance. RGS17 was found to be one of eight proteins upregulated in response to radiation treatment of MCF-7 breast cancer cells [89]. However, neither the mechanistic cause nor the consequences of this upregulation are known.

The role of high-throughput screening for RGS protein modulators

Background

As the knowledge of RGS proteins and their roles in multiple pathologies is rapidly expanding, so too are the instrumentation and technologies used to investigate the role of these proteins. The ability to rapidly gather large amounts of information about the biochemical interactions between RGS proteins and their downstream effectors has given rise to their study via high-throughput screening (HTS). The development of the HTS field has had a significant impact on the discovery of ligands for RGS proteins.

HTS & RGS inhibitors

There have been multiple attempts at the development and implementation of HTS paradigms to discover RGS inhibitors. These include the yeast-based dual luciferase assay [90,91], flow cytometry protein interaction assay (FCPIA)/multiplexed FCPIA [92–94], high-throughput fluorescence polarization assay [95], and the time-resolved fluorescence resonance energy transfer assay [96]. Each of these screens has focused on different RGS proteins from either the A/RZ or B/R4 subfamily of proteins and have laid the foundation and proof-of-principle for the validation of RGS proteins as possible drug targets.

AlphaScreen® & the first RGS17 inhibitors

Due to the emergent role of RGS17 in lung and prostate cancers, it has become an attractive target for which to develop small-molecule inhibitors. Interestingly, RGS17 is an RGS protein in which its role in cancer progression is directly related to its GAP activity through its RH domain. This allows RGS17 to be approached with established as well as novel methodologies to interrogate small-molecule libraries for inhibitors of the RGS17:Gα PPI or RGS17 GAP activity.

The development of an RGS17 inhibitor or molecular probe would work through the selective inhibition of RGS17's GAP activity. In lung and prostate tumors, RGS17 is overexpressed and, in turn, inhibits the activity of the Gα subunits. This leads to an overactivation of adenylyl cyclase and an accumulation of cAMP levels in these tumors that are overexpressing RGS17. The cAMP-PKA–CREB pathway has previously been implicated as the downstream pathway modulated by RGS17 in both lung and prostate cancers [71]. The application of an RGS17 inhibitor would work to block the GAP activity of RGS17, which would lead to an increase in the activity of the Gα subunit that interacts with adenylyl cyclase. The prolonged signaling of the Gα subunit would allow for the inhibition of adenylyl cyclase and a decrease in cAMP production within the tumorigenic tissues. The return of cAMP to basal levels in these tissues would lead to a decrease in the oncogenic activity of the oncoprotein CREB and, therefore, a decrease in tumor size and cell proliferation (Figure 2) [71].

In an attempt to improve upon 10 years of HTS screening development and to target a new RGS protein, RGS17, Mackie and coworkers adapted AlphaScreen® technology to interrogate the Gα: RGS17 PPI [97]. RGS17 was chosen based on the specific expression patterns and implication in the aforementioned disease states. This screening methodology allows for an increase in throughput while maintaining an excellent Z score exceeding 0.73, a signal-to-noise ratio >70, and a screening time of 1100 compounds per hour [97]. This

study resulted in two RGS17 inhibitors with low micromolar IC_{50} , RL-1 and RL-2. The use of AlphaScreen technology resulted in an initial hit rate of 2.5% of 1300 compounds in the primary screen, with a dose–response curve confirmation rate of 1.17% and filtered results for an overall hit rate of 0.29%. This compares favorably with the polyplexed FCPIA screen for inhibitors of multiple RGS protein interactions (initial hit rate, 1.5–3.9%; filtered results, 0.3–1.4%; and overall hit rate of 0.375%) [97]. This AlphaScreen assay format should prove powerful, as it could be readily adapted to many RGS protein interactions. AlphaScreen has been previously used to screen for numerous antagonists of PPIs including STAT-SH2 and the Hsp90 co-chaperone [98,99].

Further work has been completed in the miniaturization of the $G\alpha_o$:RGS17 PPI assay using AlphaScreen in a 1536-well format [Mackie DI *et al.*, Unpublished Data]. These findings add to the now growing literature of small-molecule RGS inhibitors.

The future of targeting RGS17

HTS has benefited from increased computing power and sensitivity of instrumentation available to researchers. This has rapidly accelerated the discovery of novel RGS modulators, however even with these advancements, there are still unmet needs. Methods are rapidly developing that will allow us to screen for RGS17 inhibitors in a cellular context, and evaluate the efficacy of putative inhibitors in culture. While the technical difficulties of measuring RGS17 inhibitors in cells are not insurmountable, they represent a first level of challenge to developing efficacious RGS17 inhibitors. Future work will need to draw on medicinal chemistry optimization to develop the structure–activity relationship of any early hit compounds as well as to optimize for *in vivo* activity. Inhibitors of the most targeted RGS protein, RGS4, were largely found to be reactive modifiers of a specific cysteine residue(s) that cause inhibition. While these inhibitors are very useful and have been used in several studies, including *in vivo*, significant effort has been devoted to developing nonreactive inhibitors [94,100,101]. Interestingly, RGS17 is not sensitive to inhibition through this mechanism, as the cysteine-reactive reagent *N*-methyl maleimide has no effect on RGS17 function [Mackie DI, Roman DL, Unpublished Data]. This could represent a promising opportunity, in that HTS targeting RGS17 will probably not identify reactive modifiers of RGS17 as inhibitors. This could provide significant advantage in the development of a potent, and reversible, RGS17 inhibitor.

Future perspective

As our understanding of the G-protein signaling pathway and its modulating proteins expands, the role of the involvement of RGS17 in multiple disease states comes into focus. With this emergence, the need for specific molecular probes to further investigate the role of RGS17 in each of these disease states *in vivo* is imperative. The identification and optimization of new chemical entities that can be used to further understand RGS17's function will improve our understanding and improve the development of RGS-directed therapeutics. The interest in all RGS proteins has benefitted the investigation of RGS17 as a druggable target, but more research is necessary to determine the full potential that RGS17 presents as a target.

Utilization of high-throughput techniques has the potential to greatly advance the field of cancer research over the next 10 years. As screening technologies progress and more and more systems are becoming amenable to the high-throughput model. Utilization of targeted libraries of drug-like molecules in these screens has the potential to increase the probability of the identification of small-molecule inhibitors that will retain drug-like activity in a cell-based system. Indeed the high-throughput model accommodates cell-based assays such that a primary screen can identify small molecules that already have activity in a cell-based

system. Utilization of these powerful tools may lead to identification of small-molecule modulators of the targets of cancer research, thereby advancing toward the ultimate goal of therapeutic development.

RGS17 has quickly emerged as a promising therapeutic target. The role of RGS17 over-expression in the progression of prostate and lung tumors alone is enough to warrant this distinction, and as the study of RGS17 has progressed additional therapeutic roles have emerged. Studies have shown the pronounced effect of RGS17 knockout *in vivo*, with drastic reduction in tumor size and progression. Therefore, an RGS17 small-molecule inhibitor has the potential to become a therapeutic in the treatment of prostate and lung cancers.

Key Term

RGS proteins	Regulators of G-protein-signaling proteins. Attenuate heterotrimeric G-protein signaling pathways via accelerating the intrinsic GTPase activity of the G α subunit
Nitric oxide synthase	a family of proteins that catalyze production of nitric oxide from L-arginine. Important modulators of vascular tone, insulin secretion, airway tone and neural development
<i>Fox04</i>	Gene that encodes for the protein Fox04, which has been demonstrated to have tumor suppressive activities. Knockdown of RGS17 resulted in an increased expression of <i>Fox04</i> , indicating RGS17 negatively regulates this tumor suppressor
<i>Hnt</i>	Gene that encodes for the protein neurotrimin, which has been reported to have tumor-suppressive activities. Knockdown of RGS17 resulted in an increased expression of <i>Hnt</i> , indicating RGS17 negatively regulates this tumor suppressor

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Executive summary

Background

- Since their discovery, G-protein-coupled receptors (GPCRs) have become the target for approximately 50% of all currently marketed therapeutics.
- Activation of the GPCR by an extracellular ligand initiates a series of coupled conformational changes, first in the GPCR and then the G α subunit.
- RGS proteins temporally regulate these conformational changes, which lead to regulation of the G-protein signaling cascade.
- GPCR signaling pathways have implicated these signaling cascades in multiple disease states that range from cancer and heart disease to immunological and neurological pathologies.

RGS subfamilies

- RGS superfamily of proteins has grown to contain eight subfamilies.
- All classical RGS proteins contain a conserved 120-amino acid domain, known as the RGS homology domain or RGS box, which is responsible for their GAP activity.
- RGS17, a member of the RZ family, is the newest discovered RGS protein and is implicated in multiple disease states.

Expression & function of RGS17

- RGS17 regulates G α_z , G $\alpha_{i/o}$ and G α_q signaling.
- Distributed almost exclusively to the brain.

Traditional RGS17 signaling

- RGS17 is involved in regulating μ -opioid receptor signaling.
- Sumoylation of RGS17 targets the protein to the membrane.
- RGS17 regulates neuronal NOS signaling.
- RGS17 regulates zinc release in response to nitric oxide donors.
- RGS17 expression levels are modulated in response to the dopamine receptors D₁ and D₂ knockdown.
- RGS17 single-nucleotide polymorphisms demonstrate a correlation to substance dependence traits.

RGS17 expression in cancer & chemoresistance mechanisms

- RGS17 is upregulated in lung and prostate cancers 8.3- and 7.5-fold, respectively, over patient-matched tissues.
- Tumor volume and mass are markedly decreased upon injection of RGS17 WT and knockdown H1299 cells into mice.
- RGS17 single-nucleotide polymorphisms were identified on chromosome 6q23–25, which correlate to an increased risk of familial lung cancer.
- Increased tumor proliferation through overexpression of RGS17 was found to be due to modulation of the cAMP-PKA–CREB pathway.

- RGS17 downregulation leads to the development of chemoresistance in ovarian carcinoma.

Role of high-throughput screening for RGS protein modulators

- The development of the high-throughput screening field has had a significant impact on the discovery of ligands for RGS proteins.
- The first RGS inhibitor was discovered using flow cytometry protein interaction assay/multiplexed flow cytometry protein interaction assay, and was an inhibitor of RGS4.
- Methods are rapidly developing that will allow researchers to screen for inhibitors of RGS proteins in a cellular context, and evaluate the efficacy of putative inhibitors in culture.

Figure 1. Classical RGS family members

Each of the four families contains the RH or RGS box domain, which is the characteristic domain of all RGS proteins. The ‘classical’ RGS family members share a conserved 50% or greater homology to the RH domain. The A/RZ family is characterized by the RH domain and a cysteine rich N-terminal domain. RGS17 is a member of the A/RZ family of RGS proteins. The B/R4 family is the simplest of the four classical families and is characterized by containing just the RH domain and an N-terminal amphiphatic helix (with the exception of RGS3, which is an R4 family member but is more complex than the other family members). The C/R7 family contains the RH domain, as well as an DEP domain and a GGL domain. Finally the D/R12 family, the largest of the classical RGS families, contains the RH domain and five accessory domains. These are the PDZ domain, the phosphotyrosine binding domain, dual Ras-binding domains and the GoLoco domain.

DEP: N-terminal dishevelled/EGL-10/pleckstrin; GGL: G-protein γ subunit-like; GoLoco: $G\alpha_{i/o}$ -Loco interaction; PDZ: PSD-95/Dlg/ZO-1; PTB: Phosphotyrosine-binding domain; RBD: Ras-binding domain; RH: RGS homology.

Figure 2. Activation of a GPCR promotes dissociation of the heterotrimeric G protein from the receptor, and simultaneous exchange of GDP for GTP in the G α subunit active site
 G $\alpha_{i/o}$ and G α_z inhibit AC and downregulate cAMP formation. RGS17 regulates G $\alpha_{i/o}$ and G α_z (as well as G α_q) by accelerating the rate of GTPase activity of the G α subunit and returning the α subunit to its inactive, GDP-bound conformation. In response to elevated levels of cAMP, PKA phosphorylates the CREB and promotes DNA transcription. Normal levels of RGS17 will modulate this cAMP-PKA-CREB signaling cascade. In lung and prostate cancers, RGS17 transcripts are increased by an average of 8.3- and 7.5-fold, respectively, over patient-matched tissues, which leads to increased transcription via CREB. Several proteins are upregulated due to this increased CREB activation, including KCIP-1, cyclin D1 and RIPK4/PKK, which leads to increased tumorigenicity via increased activity of growth, proliferative, metastasis, migratory and anti-apoptosis pathways.
 AC: Adenylate cyclase; CREB: cAMP response element binding protein; GPCR: G-protein-coupled receptor.