The chaperone protein SmgGDS interacts with small GTPases entering the prenylation pathway by recognizing the last amino acid in the CAAX motif.

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*Running title: SmgGDS-607 is a CAAX-specific binding protein

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Background: SmgGDS-607 and SmgGDS-558 regulate GTPase movement through the prenylation pathway. Results: The specificity of SmgGDS for GTPases depends on the GTPase CAAX sequence and the cellular context. Conclusion: SmgGDS-607 binds to non-prenylated GTPases that end in a leucine and enter the geranylgeranylation pathway. Significance: The identification of SmgGDS-607 as a novel CAAX-binding protein will accelerate the development of more effective cancer therapeutics.

Abstract

Ras family small GTPases localize at the plasma membrane where they can activate oncogenic signaling pathways. Understanding the mechanisms that promote membrane localization of GTPases will aid development of new therapies to inhibit oncogenic signaling. We previously reported that SmgGDS splice variants promote prenylation and trafficking of GTPases containing a C-terminal polybasic region (PBR), and demonstrated that SmgGDS-607 interacts with non-prenylated GTPases whereas SmgGDS-558 interacts with prenylated GTPases in cells. The mechanism that SmgGDS-607 and SmgGDS-558 use to differentiate between prenylated and non-prenylated GTPases has not been characterized. Here, we provide evidence that SmgGDS-607 associates with GTPases through recognition of the last amino acid in the CAAX motif. We show that SmgGDS-607 forms more stable complexes in cells with non-prenylated GTPases that will become geranylgeranylated than with non-prenylated GTPases that will become farnesylated. These binding relationships similarly occur with non-prenylated SAAX mutants. Intriguingly, farnesyltransferase inhibitors (FTIs) increase the binding of WT K-Ras to SmgGDS-607, indicating that the pharmacological shunting of K-Ras into the geranylgeranylation pathway promotes K-Ras association with SmgGDS-607. Using recombinant proteins and prenylated peptides corresponding to the C-terminal sequences of K-Ras and Rap1B, we found that both SmgGDS-607 and SmgGDS-558 directly bind the GTPase C-terminal region, but the specificity of the SmgGDS splice variants for prenylated versus non-prenylated GTPases is diminished in vitro. Finally, we present structural homology models and data from functional prediction software to define both similar and unique features of SmgGDS-607 when compared to SmgGDS-558.

Mutations and altered regulation of members of the Ras-family of GTPases can lead to uncontrollable proliferation (1, 2), metastasis (3, 4), and evasion of apoptosis (5, 6) that occur in the development and progression of cancer (7). Oncogenic signaling by GTPases occurs at the cell membrane, and is generally caused by increased expression of the GTPase (8), altered functions of
GTPase activating proteins (GAPs) or guanine nucleotide exchange proteins (GEFs) (9), or an activating mutation within the GTPase itself that interferes with GTP hydrolysis (10, 11). Due to the myriad of causes that can lead to uncontrollable signaling by a GTPase at cell membranes, research has been focused on blocking the prenylation of a GTPase and thus blocking it from reaching the plasma membrane. This therapeutic strategy has met with moderate success (12, 13).

Prenylation promotes the membrane localization of GTPases, and the addition of this hydrophobic group is the only necessary modification for GTPases that contain a C-terminal PBR to associate with membranes (14). The C-terminal region of GTPases such as K-Ras and Rap1B is known as the hypervariable region, which consists of a string of basic amino acids that make up the PBR followed by a CAAX motif (15). Addition of the prenyl moiety occurs on the cysteine of the CAAX motif (where ‘A’ represents an aliphatic amino acid), which is then further processed by cleavage of the -AAX by Rce1 and carboxyl methylation by ICMT1 (16). The type of prenylation that a GTPase will undergo is decided primarily by the last amino acid of the CAAX motif; GTPases such as Rap1B that have a CAAL motif will be geranylgeranylated, whereas GTPases such as K-Ras that have a CAAM motif will be farnesylated (17). The mechanism by which GTPases containing a PBR traffic to the plasma membrane is not well defined, with limited evidence showing direct binding of a GTPase to microtubule adaptor proteins (18), and another report showing that K-Ras uses a unique, non-exocytic pathway (19). Recently, we found that the splice variants of SmgGDS, SmgGDS-607 and SmgGDS-558, play a role in promoting prenylation and trafficking of GTPases to the cell membrane (20). However, the molecular interactions that occur during these events have not yet been characterized.

SmgGDS has been found to be a key promoter in the malignancy of multiple cancers including non-small cell lung cancer (NSCLC) (21), prostate cancer (22), and breast cancer (23). Prior to our report in 2010 (20), virtually all studies regarding SmgGDS (known as Rap1GDS1) focused on the 558-amino acid splice variant. SmgGDS-558 was originally reported as a weak GEF for multiple small GTPases that contain a PBR including Rap1 (24, 25), K-Ras (24), Rac1 (26), and RhoA (27), but more recently has been found to be a true GEF for only RhoA and RhoC (28). Structurally SmgGDS is comprised of armadillo repeats (ARMs) similar to β-catenin or karyopherin α (29), and therefore has been hypothesized to act as a scaffold-protein for multiple protein interactions (30). The ability of SmgGDS to interact with multiple GTPases and affect their nucleotide activity is most likely due to the binding of other GEFs to the scaffold-like SmgGDS, since scaffolds form a platform for protein interactions (20, 31). The C-terminal region of a GTPase is needed for interaction with SmgGDS (24, 32) and the ablation of the PBR of a GTPase will diminish its ability to complex with SmgGDS (33).

Interestingly, it was reported that SmgGDS-607 will only associate with non-prenylated GTPases whereas SmgGDS-558 will only associate with prenylated GTPases, yet the mechanism for the recognition of prenylated vs. non-prenylated GTPases has not been characterized (20). We previously found that SmgGDS-607 more effectively regulates the prenylation of GTPases that will become geranylgeranylated, such as Rap1, RhoA, and Rac1, compared to GTPases that will become farnesylated, such as K-Ras (20). This effect that SmgGDS-607 has on GTPases that will become geranylgeranylated, but not on GTPases that will become farnesylated, provides insight to its mechanism of interaction.

In this study we use K-Ras and Rap1B as model GTPases that become farnesylated and geranylgeranylated, respectively, to define the parameters that promote their physical interactions with SmgGDS splice variants. We report that SmgGDS-607 recognizes the last amino acid of a GTPase for interaction and is also involved primarily in the geranylgeranylation pathway. Our results provide evidence that SmgGDS-607 and SmgGDS-558 structurally interact with GTPases similarly in vitro, and this is in contrast to their interactions in cells. Furthermore, we provide evidence that SmgGDS-607 is involved in the alternate prenylation pathway of K-Ras in cells treated with farnesyl transferase inhibitors (FTIs).

**EXPERIMENTAL PROCEDURES**
Cell Culture, cDNA Transfection, and Drug Treatment—The HEK-293T cell line was obtained from the American Type Tissue Collection (Manassas, VA). HEK-293T cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum and antibiotics. All cDNAs were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. To inhibit prenylation, 10 μM FTI-277 (EMD Millipore, CAT344555) and GGTI-298 (EMD Millipore, CAT344555), or 15 μM mevastatin (Sigma, M2537) were added 90 min post-transfection to the cells.

cDNA Constructs—Human SmgGDS-558-HA and SmgGDS-607-HA constructs were generated as described previously (20). The majority of Myc-tagged GTPase constructs were generated as described previously (33). Myc-K-Ras CVIM was mutated by site-directed mutagenesis to change the methionine to a leucine (CVIL) and the cysteine to a serine (SVIM or SVIL) (Fig. 1A). Site-directed mutagenesis was performed using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocols, using primers purchased from Eurofins MWG/Operon. The CAAX mutant Myc-Rap1B (CQLM) was generated by site directed mutagenesis through Top Gene Technologies (St-Laurent(Montreal), Quebec, Canada).

Synthesis of K-Ras Peptides—Peptide synthesis was carried out using an automated solid-phase peptide synthesizer (PS3, Protein Technologies Inc, Memphis, TN) employing standard Fmoc/HCTU based chemistry. Synthesis began on either preloaded Fmoc-M-Wang resin or Fmoc-L-Wang resin (0.25 mmol) and the peptide chain was elongated using HCTU/N-methylmorpholine-catalyzed, single coupling steps with 4 eq of both protected amino acids and HTCU for 30 min. Following complete chain elongation, the peptide’s N-terminus was deprotected with 10% piperidine in DMF (v/v) and the presence of the resulting free amine was confirmed by ninhydrin analysis. The resin containing the peptide was washed with CH₂Cl₂, dried in vacuo overnight, weighed, and divided into two portions for further synthesis on a reduced scale. Using 0.13 mmol of peptide on resin, the free amino terminus was modified with a PEG-based linker in DMF (5 mL) using Fmoc-8-amino-3,6-dioxaoctanoic acid (77 mg, 0.20 mmol, 1.5 eq) catalyzed by DIEA (16.4 μL, 13.0 μmol) for 12 h. After installation of the linker and subsequent Fmoc deprotection, the resulting amino terminus was biotinylated in DMF (5 mL) using biotin (49.0 mg, 0.20 mmol, 1.5 eq) and HCTU (82.7 mg, 0.20 mmol, 1.5 eq) catalyzed by DIEA (16.4 μL, 13.0 μmol) for 16 h. After verifying the biotinylation was complete by ninhydrin analysis, the peptide was cleaved from the resin along with simultaneous side chain deprotection by treatment with Reagent K containing TFA (10 mL), crystalline phenol (0.5 g), 1,2-ethanediol (0.25 mL), thioanisole (0.5 mL), and H₂O (0.5 mL) for 2 h at rt. The released peptide was collected and combined with TFA washes of the resin before precipitation of the peptide in chilled Et₂O (100 mL). The crude solid peptide was collected by centrifugation, the supernatant was removed, and the resulting pellet was washed 2 times with cold Et₂O (50 mL) repeating the centrifugation and supernatant removal steps each time. The crude peptide (100 mg) was dissolved in a DMF/H₂O solution (1:5 v/v, 25 mL), applied to a semipreparative C₁₈ RP-HPLC column equilibrated in Solvent A, and washed with 10% Solvent B for 15 min. The peptide was eluted using a linear gradient (15-65% Solvent B over 1.5 h at a flow-rate of 5 mL/min). Fractions were analyzed using an analytical C₁₈ RP-HPLC column employing a linear gradient (0-100% Solvent B over 60 min at a flow-rate of 1 mL/min) and detected at 214 nm. Fractions containing peptide product of at least 90% purity were pooled and concentrated by lyophilization to yield 35 mg (27% yield) of white peptide. ESI-MS for Biotin-PEG-KKKKKKSKTKCVIM: calculated [M+3H]³⁺ = 683.66, found 683.73. ESI-MS for Biotin-PEG-KKKKKKSKTKCVIL: calculated [M+3H]³⁺ = 677.56, found 677.75

Synthesis of Rap1B (free thiol) Peptide—Solid phase peptide synthesis and purification was carried out in the same fashion as described above starting with Fmoc-L-Wang resin (0.25 mmol). No additional derivatizations were performed after peptide chain elongation was complete. After Reagent K cleavage and HPLC purification the free thiol-containing peptide resulted in 60 mg (37% yield) of white peptide. ESI-MS: calculated [M+2H]²⁺ = 943.51, found 943.68
subjected to ECL-Western blotting.

Peptide—The starting free thiol peptide (20 mg, 10.6 μmol, 1 eq) was dissolved in DMF/n-Butanol/H$_2$O (0.10% TFA) (3:1:v/v/v, 6 mL). Farnesyl bromide (15 mg, 53.5 μmol, 5 eq) was dissolved in 0.50 mL of DMF and added directly into the reaction flask that contained the dissolved peptide. Zn(OAc)$_2$·2H$_2$O (11.7 mg, 53.5 μmol, 5 eq) was then added to initiate the alkylation reaction. After 4 h the reaction was monitored by analytical RP-HPLC, purified by semipreparative C$_{18}$ RP-HPLC, and identified via ESI-TOF MS. This reaction yielded 3.4 mg (14%) of the desired alkylated peptide. ESI-MS: calculated [M+2H]$^{2+}$ = 1045.96, found 1046.04.

Synthesis of Rap1B (geranylgeranylated) Peptide—Synthesis followed the same prenylation conditions as described above using geranylgeranyl bromide (19 mg, 53.5 μmol, 5 eq). This reaction yielded 2.6 mg (12%) of the desired alkylated peptide. ESI-MS: calculated [M+2H]$^{2+}$ = 1117.37, found 1117.48.

Synthesis of Rap1B (C10) Peptide—Synthesis followed the same prenylation conditions as described above using C10-meta-Bp-Br (22.8 mg, 53.5 μmol, 5 eq) that was prepared as previously described (34-37). This reaction yielded 3.4 mg (14%) of the desired alkylated peptide. ESI-MS: calculated [M+2H]$^{2+}$ = 1079.64, found 1079.73.

Expression of Recombinant Proteins—

GST-Affinity Chromatography—Bacterial cells expressing GST-K-Ras were harvested, suspended in glutathione-binding buffer (20 mM Tris (pH 7.6), 0.15 M NaCl, 1% Triton X-100, 1 mM PMSF, bacterial protease inhibitor cocktail (Sigma), RNase (20 μg/ml), and DNase (20 μg/ml)) and lysed in a French Pressure Cell. The lysate was centrifuged (30,000 x g for 20 min) and the soluble fraction passed through a 0.45 μm filter. Bacterial lysates of GST-K-Ras CVIM or CVIL were incubated with 1.5 ml of glutathione–sepharose-4B beads (GE Healthcare) for 30 min on a rotator at 4°C. The slurry was centrifuged (1500 x g) and the resin was washed four times with 25 mL binding buffer. Proteins were batch eluted three times with 1 ml elution buffer (20 mM reduced glutathione, 100 mM Tris–HCl (pH 8.0), and 1% Triton X-100). GST-K-Ras was dialyzed into 10 mM Tris–HCl (pH 8.0), 20 mM NaCl, and 30% glycerol, and then stored at -80°C.

NTA/Ni$^{2+}$-Affinity Chromatography—

Bacterial cells expressing His-SmgGDS were suspended in a binding buffer (20 mM Tris (pH 7.9), 0.5 M NaCl, 5 mM Imidazole, bacterial protease inhibitor cocktail (Sigma), RNase (20 μg/ml), and DNase (20 μg/ml)) and lysed in a French Pressure Cell. The lysate was centrifuged (30,000 x g for 20 min) and the soluble fraction passed through a 0.45 μm filter. The filtrate was applied to an equilibrated 2 ml NTA/Ni$^{2+}$ agarose-affinity matrix (Qiagen, Valencia, CA). The column was washed with 20 ml binding buffer. SmgGDS proteins were eluted with the addition of binding buffer containing 250 mM imidazole.
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Collecting 1 mL fractions, SmgGDS-607 and SmgGDS-558 elutes in fractions 3-4.

Enhanced Chemiluminescence (ECL)-Western Blotting—Equal numbers of transfected cells were heated in Laemmli sample buffer and subjected to SDS-PAGE. The proteins were transferred as described previously (20), and immunoblotted using an antibody to SmgGDS (BD Transduction Laboratories 612511), GAPDH (Santa Cruz Biotechnology sc-32233), mouse HA antibody (Covance, MMS-101P), mouse Myc antibody (Covance, PRB-150P), rabbit Myc antibody (Sigma, C2845), rabbit HA antibody (Covance, PRB-101P), and G_1 (C-16) antibody (Santa Cruz, sc-379). Bound antibodies were visualized as described previously (20).

Analysis of Immunoprecipitates from Cultured Cells—HEK-293T cells were transiently transfected with cDNAs encoding HA-tagged SmgGDS splice variants and Myc-tagged GTPases. After 24 h, equal numbers of cells were lysed in buffer containing 0.5% Nonidet P-40 with protease and phosphatase inhibitors, and the lysates were centrifuged (2500 X g, 5 min, 4 °C). A portion of the resulting supernatants was reserved for total cell lysates, and the remainder was immunoprecipitated using mouse monoclonal HA antibody conjugated beads (Sigma, A2095), or streptavidin-sepharose 4B beads (Invitrogen, 434341) pre-bound to the indicated C-terminal K-Ras and Rap1B peptides. Immunoprecipitates, total cell lysate, and excess protein not bound to beads were subjected to ECL-Western blotting as described above.

OD values were measured by scanning the immunoblots utilizing an HP Scanjet (4850) at 600 dpi, and analyzing the scanned images using ImageJ software. OD values from Fig. 3A and Fig. 5C represent raw densitometry values. All other OD values are presented as ratios of the immunoprecipitated GTPase compared to the amount of immunoprecipitated SmgGDS.

Triton X-114 Fractionation—HEK-293T cells were lysed as described previously (39), in 1% Triton X-114 in TBS (50 mM Tris, 150 mM NaCl, pH 7.5), incubated 15 min on ice, and then centrifuged at 25,000 X g at 4 °C to remove insoluble debris. An aliquot of the cleared lysate was retained as total cell lysate. The remaining lysate was separated into aqueous and detergent fractions and examined by ECL-Western blotting as described previously (20). Proteins were detected with mouse Myc antibody to detect Myc-tagged K-Ras constructs, SmgGDS antibody (an aqueous phase loading control), and G_1 antibody (a detergent phase loading control).

Homology Modeling—Homology models of SmgGDS-608 (NCBI accession number NP_001093896) and SmgGDS-559 (NCBI accession number NP_001093893) were generated by Dr. John Sondek’s laboratory (University of North Carolina, Chapel Hill) as described previously (28). Models are displayed using PyMOL and are colored to represent the indicated armadillo (ARM) domains. An alanine is present at amino acid position #2 in both SmgGDS-608 and SmgGDS-559, and this alanine is lacking in SmgGDS-607 (NCBI accession number NP_001093897) and SmgGDS-558 (NCBI accession number NP_001093899). The presence of the alanine at position #2 is not expected to significantly change the modeled structure of the proteins.

Statistical Analyses—the means ± S.E. was measured for each value. Symbols above a column indicate a statistical comparison between the bracketed samples by One-Way ANOVA with Dunnett’s post hoc multiple comparison’s test or by Student’s t test (two-tailed), as indicated in the figure legends. p-values less than 0.05 were considered significant.

RESULTS

SmgGDS-607 is a novel CAAX-binding protein and selectively associates with non-prenylated K-Ras that becomes geranylgeranylated—To test the hypothesis that SmgGDS-607 selectively binds to GTPases that enter the geranylgeranylation pathway, K-Ras 4B (K-Ras) was used as a model. We made the indicated mutations to WT K-Ras (Fig. 1A) and then tested the ability of SmgGDS-607 and SmgGDS-558 to co-precipitate the proteins.

We found that SmgGDS-607 co-precipitates the slower migrating (non-prenylated) form of K-Ras CVIM and CVIL, and SmgGDS-558 co-precipitates the faster migrating (prenylated) form of K-Ras CVIM and CVIL (Fig. 1B, HA immunoprecipitates, lanes 1 and 2 compared to lanes 4 and 5), consistent with the previous report that SmgGDS-607 associates with non-prenylated GTPases and SmgGDS-558...
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SmgGDS-607 associates with prenylated GTPases (20). SmgGDS-607 co-precipitates significantly more with the mutant K-Ras CVIL than with WT K-Ras CVIM (Fig. 1B, HA immunoprecipitates, lane 2 compared to 1). Interestingly, SmgGDS-558 associated with both K-Ras CVIM and K-Ras CVIL, but exhibited significantly greater association with K-Ras CVIM (Fig. 1B, HA immunoprecipitates, lanes 4 and 5 and graph). These results show that SmgGDS-607 and SmgGDS-558 are novel CAAX-specific binding proteins for K-Ras, and indicate that the recognition of a GTPase by SmgGDS-607 or SmgGDS-558 requires more than just the PBR.

To test the hypothesis that SmgGDS-607 can bind WT K-Ras CVIM when the farnesylation pathway is blocked, we utilized a farnesyltransferase inhibitor (FTI-277) which is a CAAX-peptidomimetic (12). As expected, we found that SmgGDS-558 did not co-precipitate K-Ras CVIM in cells treated with a FTI (Fig. 1C, HA immunoprecipitates lane 5 compared to 6), which further indicates that SmgGDS-558 will associate with a GTPase only after prenylation. Treatment of the HEK-293T cells with a FTI did not affect the ability of SmgGDS-607 or SmgGDS-558 to co-precipitate K-Ras CVIL (Fig. 1C, HA immunoprecipitates lanes 3 compared to 4, and 7 compared to 8, respectively), consistent with the FTI not affecting GTPases that will become geranylgeranylated. Interestingly, SmgGDS-607 associated with K-Ras CVIM significantly more in cells treated with a FTI (Fig. 1C, HA immunoprecipitates lane 1 compared to 2), indicating that SmgGDS-607 can interact with a GTPase ending in a methionine but only if it will traffic through the geranylgeranylation pathway. The ability of K-Ras CVIM to interact with SmgGDS-607 in cells treated with a FTI could result from the FTI causing a larger pool of unprenylated K-Ras CVIM (Fig. 1C, Cell lysates lane 1 compared to 2). Alternatively, SmgGDS-607 might interact more with non-prenylated K-Ras CVIM in FTI-treated cells because the FTI blocks K-Ras from interacting with the farnesyltransferase, thereby forcing K-Ras CVIM to interact with proteins in the geranylgeranylation pathway (40), which includes the geranylgeranyltransferase as well as SmgGDS-607.

SmgGDS-607 recognizes the last amino acid of K-Ras for preferential binding—To test the hypothesis that SmgGDS-607 associates with K-Ras CVIM in cells treated with a FTI only when the GTPase can move through the geranylgeranylation pathway and not because there is a larger pool of K-Ras that is unprenylated, we utilized the non-prenylatable mutants K-Ras SVM and SVIL in immunoprecipitation assays (Fig. 1A and 2A). We found that SmgGDS-607 only minimally co-precipitated both K-Ras CVIM and K-Ras SVM, but strongly co-precipitated both K-Ras CVIL and K-Ras SVIL (Fig. 2A, HA immunoprecipitates lanes 1 and 5 compared to lanes 2 and 6, respectively). These results support the model that SmgGDS-607 maintains preference for K-Ras ending in leucine even when presented with a large pool of non-prenylated K-Ras ending in methionine. Treating the cells with a geranylgeranyl transferase inhibitor (GGTI-298) did not significantly change the ability of K-Ras CVIM or K-Ras CVIL to interact with SmgGDS-607 (Fig. 2A, HA immunoprecipitates lanes 3 and 4 compared to lanes 1 and 2, respectively). Taken together, these data indicate that SmgGDS-607 specifically recognizes the leucine at the end of the CAAX motif for binding. Furthermore, the association of SmgGDS-607 with K-Ras CVIM after FTI treatment indicates that regardless of the CAAX sequence, SmgGDS-607 prefers to interact with non-prenylated GTPases that are destined to interact with the geranylgeranyltransferase rather than the farnesyltransferase.

K-Ras CVIM is farnesylated and K-Ras CVIL is geranylgeranylated—To verify that the K-Ras mutant CVIL does indeed become geranylgeranylated and not farnesylated, we utilized a fractionation assay that separates proteins based on hydrophobicity (39). The Triton-X-114 fractionation assay separates proteins from a cell lysate into an aqueous (A) and detergent (D) phase. Membrane bound or hydrophobic proteins separate into the detergent phase, and cytosolic or hydrophilic proteins separate into the aqueous phase. Using cells transfected with K-Ras CVIM, we found that the majority of K-Ras CVIM fractionates into the detergent phase (Fig. 2B, top: lanes 9 and 10, Total Cell Lysate: lane 5). Treatment of these cells with either a FTI, both a FTI and a GGTI
simultaneously, or mevastatin (MEV) causes K-Ras CVIM to fractionate into the aqueous phase (Fig. 2B, top: lanes 1 and 2, 5 and 6, 7 and 8, and Total Cell Lysate: lane 1, 3 and 4). In contrast, treatment of these cells with a GGTT did not affect the fractionation of K-Ras CVIM (Fig. 2B, top: lanes 3 and 4, and Total Cell Lysate: lane 2). Using cells transfected with K-Ras CVIL, we found that the majority of K-Ras CVIL also fractionates into the detergent phase (Fig. 2B, middle: lanes 9 and 10, Total Cell Lysate: lane 10). Treatment of these cells with either a GGTT, both a FTI and a GGTT simultaneously, or MEV causes K-Ras CVIL to fractionate into the aqueous phase (Fig. 2B, middle: lanes 3 and 4, 5 and 6, 7 and 8, and Total Cell Lysate: lane 7, 8 and 9). In contrast, treatment of these cells with a FTI does not affect the fractionation of K-Ras CVIL (Fig. 2B, middle: lanes 1 and 2, and Total Cell Lysate: lane 6).

The detergent phase loading control, Gp1 subunit, fractionated primarily in the detergent phase regardless of FTI, GGTT, or mevastatin treatment indicating a large pool of this protein already pre-existed in the cells with minimal synthesis of new Gp1 protein during treatment of the cells (Fig. 2B). A longer exposure of the same immunoblots demonstrates a partial block of Gp1 prenylation in cells treated with FTI and GGTT simultaneously as well as in cells treated with mevastatin (Data not shown).

Recognition of the last amino acid in K-Ras by SmgGDS-607 is direct —The unique ability of SmgGDS-607 to recognize the last amino acid of a GTPase could be mediated by another protein that is known to participate in the prenylation pathway, such as the prenyltransferase, or perhaps an unknown protein. To identify whether the interaction between K-Ras and SmgGDS is direct we made unprenylated recombinant GST-Myc-tagged K-Ras CVIM and GST-Myc-tagged K-Ras CVIL, as well as recombinant His-tagged SmgGDS-607 and His-tagged SmgGDS-558 in order to assess their interactions. Similar to the interactions that occur in cells, we found that recombinant K-Ras CVIM did not associate with SmgGDS-607 whereas recombinant K-Ras CVIL significantly and strongly interacts with SmgGDS-607 (Fig. 3A, GST immunoprecipitation lane 1 compared to 2). We next wanted to assess the importance of the body of K-Ras in its association with SmgGDS-607; therefore we prepared biotin-labeled peptides comprised of the C-terminal PBR and CAAX motif of K-Ras CVIM or K-Ras CVIL (Fig. 3B, top). Similar to the results we obtained with the recombinant full-length K-Ras, we found that the K-Ras CVIM peptide had a weaker association than the K-Ras CVIL peptide with SmgGDS-607 (Fig. 3B, Streptavidin immunoprecipitation: 30 sec. exposure lane 1 compared to 2). These data show that the association between K-Ras CVIL and SmgGDS-607 is direct and that the isolated C-terminal region of K-Ras is able to bind to SmgGDS-607.

Unprenylated K-Ras can bind to SmgGDS-558—Surprisingly, we found that full length unprenylated recombinant K-Ras CVIM and K-Ras CVIL bound to SmgGDS-558 in vitro (Fig. 3A, GST immunoprecipitation lanes 4 and 5). This is a novel interaction between an unprenylated GTPase and SmgGDS-558 as our previous data indicated that only prenylated GTPases will associate with SmgGDS-558 in cells (20). Furthermore, we observed that the unprenylated peptides of K-Ras CVIM and CVIL also (to a lesser degree) bound to SmgGDS-558 overexpressed in HEK-293T cells (Fig. 3B, streptavidin immunoprecipitation: overnight exposure lanes 4 and 5). Taken together these data suggest that there are cellular protein(s) that bind to either the body of K-Ras or to SmgGDS-558 that help mediate the specificity of SmgGDS-558 for prenylated K-Ras. Alternatively, post translational modifications that occur only in eukaryotic cells may promote the specificity of SmgGDS-558 for prenylated K-Ras.

The association between SmgGDS-607 and a K-Ras CVIL Peptide can be competed by full length K-Ras CVIL but not K-Ras CVIM—We demonstrated that SmgGDS-607 associates well with both full length K-Ras CVIL and the K-Ras CVIL peptide, but SmgGDS-607 associates poorly with full length K-Ras CVIM and the K-Ras CVIM peptide. To confirm that these interactions are specifically defined by the last amino acid in K-Ras, we tested the prediction that the binding of SmgGDS-607 to the K-Ras CVIL peptide will be competitively inhibited by the presence of full length K-Ras CVIL, but not by full length K-Ras CVIM. To test this prediction, we utilized the biotin-K-Ras CVIL peptide in a competition assay.
with full length K-Ras CVIM or K-Ras CVIL. To define the optimal conditions for this assay, we first determined that SmgGDS-607-HA associates with the K-Ras CVIL peptide in a dose-dependent manner (Fig. 3C, top left). We determined that 1000 μg of total protein from lysates of HEK293T cells expressing SmgGDS-607-HA was an appropriate concentration to use for the competition assay, based on our dose response curve (Fig. 3C, top left and top right). We found that the addition of full length recombinant K-Ras CVIL protein, but not the addition of full length recombinant K-Ras CVIM protein, significantly prevented the association of SmgGDS-607-HA with the K-Ras CVIL peptide (Fig. 3C, top right and graph). Taken together these data demonstrate a dose-dependent association of K-Ras CVIL peptide to SmgGDS-607 that can be competitively blocked by full length K-Ras CVIL protein.

SmgGDS-607 recognizes the last amino acid in Rap1B for association—We tested whether the observed interactions of SmgGDS with specific CAAX motifs are unique to K-Ras, or conserved among multiple GTPases using Rap1B as a model. Previous reports show that SmgGDS-607 binds to Rap1B (41), Rap1A, and RhoA (20), which are GTPases that contain a PBR, a CAAX motif ending in leucine, and undergo geranylgeranylation. We tested the ability of SmgGDS-607 to co-precipitate Rap1B after mutating the last amino acid from CQLL to CQLM (Fig. 4A). SmgGDS-607 co-precipitates Rap1B CQLL strongly and Rap1B CQLM significantly less so (Fig. 4B, HA immunoprecipitates lane 1 compared to 2). There was no significant difference in the co-precipitation of SmgGDS-558 with either Rap1B CQLL or Rap1B CQLM (Fig. 4B, HA immunoprecipitates lane 4 compared to 5), which demonstrates a difference in the ability of SmgGDS-558 to recognize K-Ras CAAX-variants vs. Rap1B CAAX-variants. These data also show that SmgGDS-607 preferentially binds CAAX motifs ending in leucine in multiple GTPases.

SmgGDS-607 can bind prenylated Rap1B peptides—We next assessed the role of prenylation in the interaction of SmgGDS with a GTPase. It was previously reported that a geranylgeranylated Rap1B peptide (PGKARKKSSC), but not a un-prenylated Rap1B peptide, binds to SmgGDS-558 (32, 42). We utilized a GTPase known to be geranylgeranylated, based on our hypothesis that SmgGDS-607 will preferentially interact with a GTPase that will become geranylgeranylated. Therefore, we synthesized biotin-labeled Rap1B peptides that were either in a free thiol (F.T.) form or conjugated to farnesyl diphosphate (Far.), geranylgeranyl diphosphate (GGer.), or a photo-active C10-m-BP (C10) geranylgeranyl mimic (Fig. 5A).

Similar to data obtained using full-length GTPases in cells, we found that the non-prenylated Rap1B (F.T.) peptide co-precipitates SmgGDS-607 but not SmgGDS-558 (Fig. 5B, streptavidin immunoprecipitation lane 2 compared to 1). As expected, the geranylgeranylated Rap1B (GGer.) peptide co-precipitates SmgGDS-558 (Fig. 5B, streptavidin immunoprecipitation lane 4), but surprisingly geranylgeranylated Rap1B (GGer.) also co-precipitates SmgGDS-607 (Fig. 5B, streptavidin immunoprecipitation lane 5). We next assessed whether a farnesyl or geranylgeranylan moiety conjugated to Rap1B affects association to SmgGDS. SmgGDS-607 strongly co-precipitates with both the farnesylated Rap1B (Far.) and geranylgeranylated Rap1B (GGer.) peptides whereas, interestingly, SmgGDS-558 co-precipitates less strongly with both prenylated peptides (Fig. 5C, streptavidin immunoprecipitation lanes 6 and 7 compared to 10 and 11). These are the first findings that identify a prenylated GTPase peptide binding to SmgGDS-607.

In order to test the hypothesis that a prenylated Rap1B peptide is directly binding to SmgGDS-607, we utilized a photo-activatable geranylgeranyl mimic (C10) which was conjugated to a Rap1B peptide (Fig. 5A). The benzophenone-containing prenyl-group is a photophore that is chemically stable and activated by UV light which cross-links the conjugated species (Rap1B peptide) to proteins that directly interact with the conjugated substrate (SmgGDS-607) (43-46). We found that the Rap1B (C10) peptide crosslinking to SmgGDS-607 increased with greater UV exposure (Fig. 5D). The control lane shows a weak association between SmgGDS-607 and the C10 peptide at a lower molecular weight comparable to the SmgGDS-607 whole cell lysate input (Fig. 5D, lane 5 compared to 7) which
indicates binding, but not cross-linking. Taken together, these results indicate a novel interaction between a prenylated GTPase peptide and SmgGDS-607, which differs from our previous data that SmgGDS-607 will only recognize a non-prenylated GTPase in cells (20).

SmgGDS-607 and SmgGDS-558 share similar predicted protein structures as well as similar functional domains with some unique differences—SmgGDS-607 and SmgGDS-558 are armadillo proteins that we previously described as consisting of ARM domains A–M (20). SmgGDS-558 is a splice variant of SmgGDS-607 and is lacking the ‘C’ ARM domain (Fig. 6A, yellow shaded area). We analyzed homology models of the 608- and 559-amino acid forms of SmgGDS. These forms of SmgGDS contain an additional alanine at position 2 compared to the 607- and 558-amino acid forms of SmgGDS. The insertion of this alanine should have minimal or no effect on the structure of SmgGDS-608 compared to SmgGDS-607, and SmgGDS-559 compared to SmgGDS-558. Thus, it is reasonable to assume that the predicted structures of SmgGDS-608 and SmgGDS-559 are similar to those of SmgGDS-607 and SmgGDS-558, respectively. These homology models predict that the N-terminal region consisting of ARMs A and B is structurally very different in SmgGDS-608 compared to SmgGDS-607, and SmgGDS-559 compared to SmgGDS-558. In contrast, SmgGDS-608 and SmgGDS-559 are predicted to share very similar structures in the region consisting of ARMs D–M (Fig. 6A, green shaded area). This region contains the electronegative patch in SmgGDS that interacts with the positively charged C-terminal PBR of a GTPase (28).

Utilizing the I-TASSER server (47-49), we analyzed the function of SmgGDS-607 and SmgGDS-558 through COFACTOR software. The COFACTOR software utilizes functional libraries to assess the predicted protein structure’s match to functional sites and homologies of other known protein complexes. Table 1 shows the top five predicted enzymatic homologs of the SmgGDS splice variants using known enzyme-substrate complexes. Similar predicted functions between SmgGDS-607 and SmgGDS-558 include the functions of the V-Type ATPase subunit H, PME-1 and PP2A core enzyme complex, and Immunoglobulin A1 protease (Table 1).

Interestingly, SmgGDS-558, but not SmgGDS-607, shared an enzymatic homology with a protein farnesyltransferase bound to a caged TCVIM peptide and farnesyl diphosphate. This difference between SmgGDS-558 and SmgGDS-607 is consistent with SmgGDS-558 uniquely recognizing prenylated GTPases.

**DISCUSSION**

Our results indicate that the binding of a GTPase to SmgGDS-607 is based not only on the PBR of the GTPase (30) but also by the last amino acid of the GTPase. This finding indicates that SmgGDS-607 is a novel CAAX-binding protein and provides new insights into the regulation of GTPases before their entry into the prenylation pathway. Furthermore, our results show that SmgGDS-607 preferentially binds GTPases that will become geranylgeranylated, rather than GTPases that will become farnesylated. These findings identify SmgGDS-607 as a target for cancer therapeutics, especially therapies aimed at inhibiting protein prenylation.

Our proposed model shows that SmgGDS-607 will associate with GTPases that end in a leucine and will move through the geranylgeranylation pathway, but not with a GTPase that ends in a methionine and will move through the farnesylation pathway (Fig. 6B). Previous data (20) along with the data presented in this study support the idea that SmgGDS-607 acts as a storage protein that binds the non-prenylated GTPase until the geranylgeranyltransferase (GGTase) can process it. Storage of a non-prenylated GTPase by SmgGDS-607 is an ideal way to regulate the entrance of a GTPase into the geranylgeranylation pathway. SmgGDS-607 may promote the trafficking of a GTPase to the GGTase similar to the interaction of the Rab Escort Protein-1 with rab proteins (50, 51). In this case, SmgGDS-607 would recognize a newly synthesized GTPase ending in a leucine and present it to the GGTase for prenylation. Our previous studies indicate that certain signals such as nucleotide exchange (20) or phosphorylation (41) of a GTPase will determine the ability of a GTPase to bind SmgGDS-607 and/or be released to the GGTase.

We found that mutating the C-terminal residue of K-Ras from a methionine to a leucine significantly increases the binding of K-Ras to
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SmgGDS-607. This leucine substitution causes K-Ras to become geranylgeranylated instead of farnesylated, supporting our model that SmgGDS-607 prefers binding small GTPases that have a C-terminal leucine and will become geranylgeranylated.

Quite surprisingly, we found that treatment with a FTI increased the co-precipitation of complexes consisting of WT K-Ras and SmgGDS-607 from cells. This result might seem perplexing, because we would not expect SmgGDS-607 to bind WT K-Ras due to the presence of the C-terminal methionine. However, it is well known that the presence of an FTI causes WT K-Ras to have reduced interactions with the farnesyltransferase (FTase) and increased interactions with the GGTase, resulting in the geranylgeranylation of K-Ras. It is intriguing to speculate that complexes of K-Ras and SmgGDS-607 can be isolated from FTI-treated cells because K-Ras has increased interaction with the GGTase in these cells. If SmgGDS-607 acts as a scaffold that binds the GGTase, then SmgGDS-607 would be expected to bind the GGTase when it is interacting with K-Ras in FTI-treated cells. In this case, SmgGDS-607 will not directly bind K-Ras (due to the presence of the C-terminal methionine), but instead SmgGDS-607 will bind the complex consisting of the GGTase and K-Ras.

Taken together, our findings support the model that SmgGDS-607 directly binds GTPases that have the CAAL motif (as indicated by our studies using recombinant proteins), but SmgGDS-607 can also indirectly bind GTPases that have the CAAM motif if they are associated with the GGTase (as in the case of K-Ras in FTI-treated cells). This model predicts that SmgGDS-607 will physically interact with both the GTPase and the GGTase. However, our initial studies trying to immunoprecipitate the GGTase I-β subunit with SmgGDS-607 or SmgGDS-558 either in the presence or absence of a GTPase have yielded no associations (data not shown). We have also tried to probe our immunoblots of immunoprecipitated complexes using antibodies that recognize the FTase or GGTase with no success.

The interesting finding that K-Ras will associate with SmgGDS-607 in cells treated with a FTI provides novel insight into the mechanism of alternate prenylation and has potential therapeutic implications. FTIs were developed to block farnesylation of oncogenic Ras and prevent it from reaching the plasma membrane where its downstream target proteins are localized (52, 53). Although promising initially, the main limitation of FTIs was the lack of effectiveness in clinical trials (54, 55). Interestingly, it was found that in cells treated with FTIs, K-Ras could become alternatively geranylgeranylated and still continue to promote oncogenic signaling from the membrane (40). More success came from utilizing FTIs simultaneously with GGTIs, however toxicity then became an issue (56, 57).

The association of K-Ras with SmgGDS-607 in cells treated with FTIs suggests that SmgGDS-607 might participate in the alternative geranylgeranylation of K-Ras in the presence of FTIs. If SmgGDS-607 regulates the entry of K-Ras into the geranylgeranylation pathway in FTI-treated cells, then inhibiting interactions of SmgGDS-607 with K-Ras (either through down-regulation or inactivation of SmgGDS-607) could provide a novel way to inhibit K-Ras geranylgeranylation when FTIs are administered. Thus, SmgGDS-607 could be a novel target for combination therapeutics, as initial studies show that knockdown of SmgGDS-607 alone is not deleterious in breast and lung cancers (20, 23).

However, it is important to note that the association of K-Ras with SmgGDS-607 in FTI-treated cells could simply be an ancillary result, whereby K-Ras can become geranylgeranylated regardless of its association with SmgGDS-607. More tests are needed to define the function of SmgGDS-607 in the alternative geranylgeranylation of K-Ras when farnesylation is inhibited.

SmgGDS-558 differs from SmgGDS-607 because SmgGDS-558 is restricted to binding prenylated GTPases in cells (20). Interestingly, the analysis of predicted functional properties between SmgGDS-558 and SmgGDS-607 identified that the structure of SmgGDS-558 (but not SmgGDS-607) has enzymatic homology with a FTase that has a caged K-Ras peptide and farnesyl pyrophosphate bound, which supports the observation that SmgGDS-558 will functionally bind prenylated GTPases (Table 1).

We found that in cells, SmgGDS-558 binds prenylated K-Ras that has either a methionine or leucine as the last amino acid, but interestingly SmgGDS-558 co-precipitates...
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significantly more K-Ras CVIM than K-Ras CVIL. The most likely explanation for this finding is that the prenylated form of K-Ras is generated more from K-Ras CVIM than from K-Ras CVIL in the cells, thereby providing more prenylated K-Ras for SmgGDS-558 to co-precipitate from the cells. This possibility is supported by our finding that a higher ratio of prenylated to non-prenylated K-Ras is generated when K-Ras CVIM is expressed than when K-Ras CVIL is expressed (Fig. 1C, Cell lysates: lane 6 compared to lane 8). A less likely explanation for SmgGDS-558 co-precipitating significantly more K-Ras CVIM than K-Ras CVIL is that SmgGDS-558 prefers to associate more with farnesylated K-Ras (which is generated from K-Ras CVIM) than with geranylgeranylated K-Ras (which is generated from K-Ras CVIL). Finally, a third potential explanation for the preference of SmgGDS-558 for K-Ras CVIM is that SmgGDS-558 interacts with prenylated K-Ras before the CAAX is cleaved from the CAAX motif by Rce1. In this speculative model, SmgGDS-558 binds prenylated GTPases that still retain their CAAX motif, with a preference for a CAAX motif ending in methionine rather than leucine. If these last two explanations are correct, we would expect SmgGDS-558 to exhibit the same binding preferences for the Rap1B CAAX variants as those shown for the K-Ras CAAX variants. However, we found that SmgGDS-558 co-precipitates equal amounts of Rap1B CQLM and Rap1B CQLL from cells (Fig. 4), suggesting that neither the type of isoprenoid modification (farnesyl versus geranylgeranyl) nor the last amino acid in the CAAX motif (methionine versus leucine) is dictating the specificity of SmgGDS-558 for the prenylated GTPases. Instead, the ratio of prenylated to non-prenylated GTPase seems to be the most likely indicator for interaction with SmgGDS-558. This conclusion is supported by our finding that Rap1B CQLM and Rap1B CQLL generate the same ratios of prenylated to non-prenylated Rap1B (Fig. 4B, Cell lysates: lane 4 compared to lane 5), and are equally co-precipitated by SmgGDS-558.

The ability of SmgGDS-558 to associate with both farnesylated and geranylgeranylated GTPases in cells supports the idea that this splice variant may play a greater role than SmgGDS-607 in the trafficking of oncogenic GTPases. Indeed, previous studies indicate that the malignant phenotype of NSCLC and breast cancer is decreased more by the RNAi-mediated knockdown of SmgGDS-558 than by SmgGDS-607 (20, 23). However, it is possible that the RNAi-mediated knockdown of SmgGDS-607 did not deplete SmgGDS-607 protein levels enough in these experiments to produce a functional effect. The development of small molecule inhibitors for both SmgGDS splice variants will aid further studies of their unique functions (20, 23).

We made the unexpected discovery that the preference of SmgGDS-558 for prenylated GTPases is lost when SmgGDS-558 is allowed to interact with GTPases in vitro. This conclusion is supported by our observation that even though SmgGDS-558 co-precipitates only the prenylated forms of WT K-Ras and K-Ras-CVIL in cells, SmgGDS-558 will bind to non-prenylated WT K-Ras and K-Ras-CVIL in recombinant systems in vitro. Additionally, we show that SmgGDS-558 will bind a non-prenylated C-terminal K-Ras peptide. Consistent with these findings, it was previously reported that SmgGDS-558 can induce guanine nucleotide exchange by the non-prenylated form of RhoA in a recombinant system, but promotes guanine nucleotide exchange by only prenylated RhoA in cells (28). These findings indicate that SmgGDS-558 prefers prenylated GTPases only when interacting with the GTPases in a cellular system. Post-translational modifications or unknown protein partners may mediate the interaction of SmgGDS-558 with prenylated GTPases in cells, and the loss of these post-translational modifications or protein partners in recombinant systems may allow SmgGDS-558 to interact with non-prenylated GTPases in vitro.

Another interesting finding was the strong association of a prenylated Rap1B peptide to SmgGDS-607, which is surprising considering our previous model that SmgGDS-607 only binds non-prenylated GTPases. This novel discovery led us to assess whether this interaction was direct, and indeed using cross-linking we show that a prenylated Rap1B peptide covalently binds to SmgGDS-607. It is not clear why SmgGDS-607 directly binds the prenylated Rap1B C-terminal peptide in vitro, when SmgGDS-607 is restricted to binding only non-prenylated full length Rap1B in cells. The prenylated peptide might bind to SmgGDS-607 through non-specific hydrophobic
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interactions that arise in the in vitro system. Alternatively, the prenylated peptide might specifically bind a hydrophobic pocket in SmgGDS-607 that is accessible to the prenylated peptide in vitro, but is inaccessible to full-length prenylated Rap1B in a cellular context. Further testing will be needed to define the reasons for this surprising result.

The homology models help explain several of the interactions we have observed in this study. According to these models, SmgGDS-608 and SmgGDS-559 are structurally very similar in ARMs D – M, which contains the electronegative patch that binds the PBR of a GTPase (28). We propose that when SmgGDS and a GTPase are allowed to interact in vitro without the presence of other proteins, the GTPases that are non-prenylated can associate with both SmgGDS splice variants through an electrostatic interaction between the PBR and the electronegative patch in ARMs D – M. This prediction is supported by our finding that both SmgGDS-607 and SmgGDS-558 bind non-prenylated K-Ras in recombinant systems.

The very different structure predicted for ARMs A and B in SmgGDS-608 compared to SmgGDS-559 might explain why the two SmgGDS splice variants differ in their ability to bind non-prenylated versus prenylated GTPases in cells. We propose that the different N-terminal structures of the two SmgGDS splice variants might cause them to have unique post-translational modifications or to interact with different protein partners in cells. These post-translational modifications and/or interactions with cellular protein partners may define the specificity of SmgGDS-607 for non-prenylated GTPases, and SmgGDS-558 for prenylated GTPases that is observed only in cellular systems. Thus, in vitro with no other proteins available, the splice variants would lose their ability to differentiate between prenylated and non-prenylated GTPases, and their interactions with GTPases would be dictated mainly by the electronegative patch in ARMs D – M.

This study is the first report that SmgGDS-607 binds GTPases by recognizing the last amino acid of the GTPase. SmgGDS-607 is shown to be involved specifically in the geranylgeranylation pathway for GTPases. Although direct, the associations of SmgGDS-607 and SmgGDS-558 with GTPases differ between cells and in vitro, suggesting that these interactions are regulated by processes that uniquely occur in intact cellular systems. Further studies should be focused on developing small molecule inhibitors for both splice variants of SmgGDS and assessing their potential therapeutic use.
REFERENCES

1. Lloyd AC. Ras versus cyclin-dependent kinase inhibitors. Curr Opin Genet Dev. 1998;8(1):43-8.
2. Yoshida Y, Kawata M, Miura Y, Musha T, Sasaki T, Kikuchi A, et al. Microinjection of smg/rap1/krev-1 p21 into swiss 3T3 cells induces DNA synthesis and morphological changes. Mol Cell Biol. 1992;12(8):3407-14.
3. Janda E, Lehmann K, Killisch I, Jechlinger M, Herzig M, Downward J, et al. Ras and TGFβ cooperatively regulate epithelial cell plasticity and metastasis dissection of ras signaling pathways. J Cell Biol. 2002;156(2):299-314.
4. Bailey CL, Kelly P, Casey PJ. Activation of Rap1 promotes prostate cancer metastasis. Cancer Res. 2009;69(12):4962-8.
5. Mayo MW, Wang C, Cogswell PC, Rogers-Graham KS, Lowe SW, Der CJ, et al. Requirement of NF-κB activation to suppress p53-independent apoptosis induced by oncogenic ras. Science. 1997;278(5344):1812-5.
6. Downward J. Ras signalling and apoptosis. Curr Opin Genet Dev. 1998;8(1):49-54.
7. Malumbres M, Barbacid M. RAS oncogenes: The first 30 years. Nature Reviews Cancer. 2003;3(6):459-65.
8. Sahai E, Marshall CJ. RHO–GTPases and cancer. Nature Reviews Cancer. 2002;2(2):133-42.
9. Vigil D, Cherfils J, Rossman KL, Der CJ. Ras superfamily GEFs and GAPs: Validated and tractable targets for cancer therapy? Nature Reviews Cancer. 2010;10(12):842-57.
10. Viola MV, Fromowitz F, Oravez S, Deb S, Finkel G, Lundy J, et al. Expression of ras oncogene p21 in prostate cancer. N Engl J Med. 1986;314(3):133-7.
11. Boguski MS, McCormick F. Proteins regulating ras and its relatives. Nature. 1993;366(6456):643-54.
12. Lerner EC, Qian Y, Blaskovich MA, Fossum RD, Vogt A, Sun J, et al. Ras CAAX peptidomimetic FTI-277 selectively blocks oncogenic ras signaling by inducing cytoplasmic accumulation of inactive ras-raf complexes. Journal of Biological Chemistry. 1995 November 10;270(45):26802-6.
13. Konstantinopoulos PA, Karamouzis MV, Papavassiliou AG. Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. Nature Reviews Drug Discovery. 2007;6(7):541-55.
14. Hancock JF, Paterson H, Marshall CJ. A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21 ras to the plasma membrane. Cell. 1990;63(1):133-9.
15. Cadwallader K, Paterson H, Macdonald S, Hancock J. N-terminally myristoylated ras proteins require palmitoylation or a polybasic domain for plasma membrane localization. Mol Cell Biol. 1994;14(7):4722-30.
16. Winter-Vann AM, Casey PJ. Post-prenylation-processing enzymes as new targets in oncogenesis. Nature reviews Cancer. 2005;5(5):405-12.
17. Moores SL, Schaber M, Mosser S, Rands E, O’hara M, Garsky V, et al. Sequence dependence of protein isoprenylation. J Biol Chem. 1991;266(22):14603-10.
18. Thissen JA, Gross JM, Subramanian K, Meyer T, Casey PJ. Prenylation-dependent association of k- ras with microtubules EVIDENCE FOR A ROLE IN SUBCELLULAR TRAFFICKING. J Biol Chem. 1997;272(48):30362-70.
19. Apolloni A, Prior IA, Lindsay M, Parton RG, Hancock JF. H-ras but not K-ras traffics to the plasma membrane through the exocytic pathway. Mol Cell Biol. 2000;20(7):2475.
20. Berg TJ, Gastonguay AJ, Lorimer EL, Kuhnmuench JR, Li RS, Fields AP, et al. Splice variants of SmgGDS control small GTPase prenylation and membrane localization. J Biol Chem. 2010 Nov;285(46):35255-66.
21. Tew GW, Lorimer EL, Berg TJ, Zhi HY, Li RS, Williams CL. SmgGDS regulates cell proliferation, migration, and NF-kappa B transcriptional activity in non-small cell lung carcinoma. J Biol Chem. 2008 Jan;283(2):963-76.
SmGDS-607 is a CAAX-specific binding protein

22. Zhi H, Yang XJ, Kuhnmuench J, Berg T, Thill R, Yang H, et al. SmgGDS is up-regulated in prostate carcinoma and promotes tumour phenotypes in prostate cancer cells. J Pathol. 2009 Feb;217(3):389-97.

23. Hauser AD, Bergom B, Schuld NJ, Chen X, Lorimer EL, Huang J, et al. The SmgGDS splice variant SmgGDS-558 is a key promoter of tumor growth and RhoA activation in breast cancer. Mol Cancer Res. November 6, 2013; doi: 10.1158/1541-7786.MCR-13-0362.

24. Mizuno T, Kaibuchi K, Yamamoto T, Kawamura M, Sakoda T, Fujioka H, et al. A stimulatory GDP/GTP exchange protein for smg p21 is active on the post-translationally processed form of c-ki-ras p21 and rhoA p21. Proceedings of the National Academy of Sciences. 1991 August 01;88(15):6422-6.

25. Yamamoto T, Kaibuchi K, Mizuno T, Hiroyoshi M, Shirataki H, Takai Y. Purification and characterization from bovine brain cytosol of proteins that regulate the GDP/GTP exchange reaction of smg p21s, ras p21-like GTG-binding proteins. J Biol Chem. 1990;265(27):16626-34.

26. Hiraoaka K, Kaibuchi K, Ando S, Musha T, Takaishi K, Mizuno T, et al. Both stimulatory and inhibitory GDP/GTP exchange proteins, smg GDS and rho GDI, are active on multiple small GTP-binding proteins. Biochem Biophys Res Commun. 1992;182(2):921-30.

27. Isomura M, Kikuchi A, Ohga N, Takai Y. Regulation of binding of rhoB p20 to membranes by its specific regulatory protein, GDP dissociation inhibitor. Oncogene. 1991;6(1):119-24.

28. Hamel B, Monaghan-Benson E, Rojas RJ, Temple BRS, Marston DJ, Burridge K, et al. SmgGDS is a guanine nucleotide exchange factor that specifically activates RhoA and RhoC. J Biol Chem. 2011 Apr;286(14) 12141-48.

29. Peifer M, Berg S, Reynolds AB. A repeating amino acid motif shared by proteins with diverse cellular roles. Cell. 1994;76(5):789-91.

30. Williams CL. The polybasic region of ras and rho family small GTPases: A regulator of protein interactions and membrane association and a site of nuclear localization signal sequences. Cell Signal. 2003 Dec;15(12):1071-80.

31. Shin HJ, Lee CH, Cho IH, Kim Y, Lee Y, Kim IA, et al. Electrospun PLGA nanofiber scaffolds for articular cartilage reconstruction: Mechanical stability, degradation and cellular responses under mechanical stimulation in vitro. Journal of Biomaterials Science, Polymer Edition. 2006;17(1-2):103-19.

32. Kotani K, Kikuchi A, Doi K, Kishida S, Sakoda T, Kishi K, et al. The functional domain of the stimulatory GDP/GTP exchange protein (smg GDS) which interacts with the C-terminal geranylgeranylated region of rap1/krev-1/smg p21. Oncogene. 1992, Sep;7(9):1699-704.

33. Lanning CC, Ruiz-Velasco R, Williams CL. Novel mechanism of the co-regulation of nuclear transport of SmgGDS and rac1. J Biol Chem. 2003 Apr;278(14):12495-506.

34. Turek TC, Gaon I, Gamache D, Distefano MD. Synthesis and evaluation of benzophenone-based photoaffinity labeling analogs of prenyl pyrophosphates containing stable amide linkages. Bioorg Med Chem Lett. 1997;7(16):2125-30.

35. Turek TC, Gaon I, Distefano MD, Strickland CL. Synthesis of farnesyl diphosphate analogues containing ether-linked photoactive benzenophenes and their application in studies of protein prenyltransferases. J Org Chem. 2001;66(10):3253-64.

36. Kale TA, Raab C, Yu N, Dean DC, Distefano MD. A photoactivatable prenylated cysteine designed to study isoprenoid recognition. J Am Chem Soc. 2001;123(19):4373-81.

37. Turek TC, Gaon I, Distefano MD. Synthesis and rapid purification of 32P-labeled photoactive analogs of farnesyl pyrophosphate. J Labelled Compd Radiopharmaceut. 1997;39(2):139-46.

38. Maresso AW, Barbieri JT. Expression and purification of two recombinant forms of the type-III cytotoxin, pseudomonas aeruginosa ExoS. Protein Expr Purif. 2002;26(3):432-7.

39. Hancock JF. Prenylation and palmitoylation analysis. Meth Enzymol. 1995;255:237-45.

40. Whyte DB, Kirschmeier P, Hockenberry TN, Nunez-Oliva I, James L, Catino JJ, et al. K-and N-ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J Biol Chem. 1997;272(22):14459-64.
SmgGDS-607 is a CAAX-specific binding protein

41. Ntanie E, Gonyo P, Lorimer EL, Hauser AD, Schuld N, McAllister D, et al. An adenosine-mediated signaling pathway suppresses prenylation of the GTPase Rap1B and promotes cell scattering. Science signaling. 2013;6(277):ra39.

42. Shirataki H, Kaibuchi K, Hiroyoshi M, Isomura M, Araki S, Sasaki T, et al. Inhibition of the action of the stimulatory GDP/GTP exchange protein for smg p21 by the geranylgeranylated synthetic peptides designed from its C-terminal region. Journal of Biological Chemistry. 1991 November 05;266(31):20672-7.

43. Kyro K, Manandhar SP, Mullen D, Schmidt WK, Distefano MD. Photoaffinity labeling of ras converting enzyme using peptide substrates that incorporate benzoylphenylalanine (bpa) residues: Improved labeling and structural implications. Bioorg Med Chem. 2011 12/15;19(24):7559-69.

44. Dorman G, Prestwich GD. Benzophenone photophores in biochemistry. Biochemistry (N Y ). 1994;33(19):5661-73.

45. Chowdhry V, Westheimer F. Photoaffinity labeling of biological systems. Annu Rev Biochem. 1979;48(1):293-325.

46. S Vervacke J, Wang Y, D Distefano M. Photoactive analogs of farnesyl diphosphate and related isoprenoids: Design and applications in studies of medicinally important isoprenoid-utilizing enzymes. Curr Med Chem. 2013;20(12):1585-94.

47. Roy A, Yang J, Zhang Y. COFACTOR: An accurate comparative algorithm for structure-based protein function annotation. Nucleic Acids Res. 2012;40(W1):W471-7.

48. Roy A, Kucukural A, Zhang Y. I-TASSER: A unified platform for automated protein structure and function prediction. Nature protocols. 2010;5(4):725-38.

49. Zhang Y. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics. 2008;9(1):40.

50. Alexandrov K, Horiuchi H, Steele-Mortimer O, Seabra M, Zerial M. Rab escort protein-1 is a multifunctional protein that accompanies newly prenylated ras proteins to their target membranes. EMBO J. 1994;13(22):5262.

51. Stenmark H, Olkkonen VM. The rab GTPase family. Genome Biol. 2001;2(5):S3007.

52. Basso AD, Kirschmeier P, Bishop WR. Thematic review series: Lipid posttranslational modifications. Farnesyl transferase inhibitors. J Lipid Res. 2006;47(1):15-31.

53. Law BK, Nørgaard P, Moses HL. Farnesyltransferase inhibitor induces rapid growth arrest and blocks p70s6k activation by multiple stimuli. J Biol Chem. 2000;275(15):10796-801.

54. Rao S, Cunningham D, De Gramont A, Scheithauer W, Smakal M, Humblet Y, et al. Phase III double-blind placebo-controlled study of farnesyl transferase inhibitor R115777 in patients with refractory advanced colorectal cancer. Journal of Clinical Oncology. 2004;22(19):3950-7.

55. Macdonald JS, McCoy S, Whitehead RP, Iqbal S, Wade III JL, Giguere JK, et al. A phase II study of farnesyl transferase inhibitor R115777 in pancreatic cancer: A southwest oncology group (SWOG 9924) study. Invest New Drugs. 2005;23(5):485-7.

56. Sun J, Qian Y, Hamilton AD, Sebti SM. Both farnesyltransferase and geranylgeranyltransferase I inhibitors are required for inhibition of oncogenic K-ras prenylation but each alone is sufficient to suppress human tumor growth in nude mouse xenografts. Oncogene. 1998 Mar;16(11):1467-73.

57. Lobell RB, Omer CA, Abrams MT, Bhimnathwala HG, Brucker MJ, Buser CA, et al. Evaluation of farnesyl: Protein transferase and geranylgeranyl: Protein transferase inhibitor combinations in preclinical models. Cancer Res. 2001;61(24):8758-68.
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FOOTNOTES

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6The abbreviations used are:  PBR, poly-basic region; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; NSCLC, non-small cell lung carcinoma; ARM, armadillo; FTI, farnesyltransferase inhibitor; GGTI, geranylgeranyltransferase inhibitor; GGer., geranylgeranyl; F.T., free thiol; Far., farnesyl; GGTase, geranylgeranyltransferase; FTase, farnesyltransferase; MEV, mevastatin.

FIGURE LEGENDS

Table 1: Structure-based functional predictions of SmgGDS-607 and SmgGDS-558. Analysis of SmgGDS-607 and SmgGDS-558 utilizing the I-TASSER structural and functional prediction software which reports COFACTOR: Structure-based functional predictions. The top five proteins that are shown share similar structural active sites to either SmgGDS-607 or SmgGDS-558. CscoreEC is the confidence score for the Enzyme Classification (EC) number prediction. CscoreEC values range between [0-1]; a higher score indicates a more reliable EC number prediction. TM-score is a measure of global structural similarity between query and template protein where scores <0.17 correspond to a random similarity. PDB Hit is the protein database signifier.

Figure 1: SmgGDS-607 is a novel CAAX-binding protein and associates with non-prenylated small GTPases that will become geranylgeranylated. A. WT K-Ras 4B has a CAAX motif of CVIM which allows for the protein to become farnesylated. The last amino acid of K-Ras was mutated to a leucine in order to promote geranylgeranylation (40). The cysteine of the CAAX motif was mutated to a serine in order to inhibit prenylation of K-Ras (20). B and C. HEK-293T cells were transfected with a cDNA encoding SmgGDS-607-HA, SmgGDS-558-HA, or the HA vector plus a cDNA encoding the Myc-tagged WT K-Ras CVIM, CVIL, or a Myc vector. 90 min post transfection the cells were treated with 10 μM FTI-277 (odd lanes, C) or vehicle (even lanes, C). After 24 h, cells were lysed, and an aliquot of each lysate was subjected to ECL-Western blotting using Myc antibody (Cell Lysates). The remaining volume of each cell lysate was immunoprecipitated with HA antibody, followed by ECL-Western blotting using HA antibody and Myc antibody (HA Immunoprecipitates). Results are shown as the optical density of IP Myc-K-Ras divided by the optical density of IP SmgGDS-HA and are the mean ± S.E. of four (B) and three (C) independent experiments. (ns, not significant; *, p<0.05; **, p<0.01 by Student’s t test).
**Figure 2:** K-Ras CVIM is farnesylated whereas K-Ras CVIL is geranylgeranylated, and SmgGDS-607 recognizes the last amino acid in the CAAX motif for binding. A. SmgGDS-607-HA was expressed in HEK-293T cells with Myc-tagged WT K-Ras CVIM, CVIL, or their non-prenylatable mutants K-Ras SVIM and K-Ras SVIL, or a Myc vector. 90 min post-transfection the cells were treated with either GGTI (15 μM) or vehicle. After 24 h, cells were lysed, and an aliquot of each lysate was subjected to ECL-Western blotting using Myc antibody (Cell Lysates). The remaining volume of each cell lysate was immunoprecipitated with HA antibody, and the immunoprecipitates were immunoblotted using HA and Myc antibodies (HA Immunoprecipitates). Results are shown as the optical density of IP Myc-K-Ras divided by the optical density of IP SmgGDS-HA and are the mean ± S.E. of three independent experiments. (ns, not significant; *, p<0.01 by One-Way ANOVA with Dunnett’s post-test compared to the control value of Myc-K-Ras CVIM pulled down lane 1). B. HEK-293T cells were transfected with a cDNA encoding either Myc-tagged WT K-Ras CVIM (top) or mutant K-Ras CVIL (bottom). 90 min post transfection cells were treated with either FTI-277 (10 μM), GGTI-298 (10 μM), both FTI and GGTI, mevastatin (10 μM), or vehicle control. After 24 h, the cells were lysed, and an aliquot of each lysate was subjected to ECL-Western blotting using Myc antibody (Total Cell Lysates). The remaining volume of each cell lysate was subjected to Triton X-114 fractionation, and equal volumes of the aqueous phase, detergent phase, or total cell lysate were immunoblotted using Myc antibody, antibody to SmgGDS (aqueous phase marker), or antibody to G₁₁₁ subunit (detergent phase marker).

**Figure 3:** SmgGDS-607 binds directly and specifically to K-Ras CVIL, and SmgGDS-558 binds unprenylated WT K-Ras CVIM or CVIL in vitro. A. 100 ng of recombinant His-tagged SmgGDS-607 and SmgGDS-558 were allowed to interact with 250 ng of recombinant GST-Myc-tagged full length K-Ras CVIM or K-Ras CVIL proteins pre-bound to glutathione sepharose 4B beads. The complexes were isolated by pelleting the beads, and subjected to ECL-Western blotting using a SmgGDS antibody. Results are shown as the optical density of the SmgGDS protein signal in the immunoblots and are the mean ± S.E. of three independent experiments. (ns, not significant; **, p<0.01 by Student’s t test). B. Biotin-PEG₉-K-Ras CVIM or CVIL peptides with the sequence KKKKKSKTKCVIM/L (top) were pre-bound (10 μg) to streptavidin beads and allowed to interact with HEK-293T cell lysates expressing either SmgGDS-607-HA, SmgGDS 558-HA, or HA Vector. The complexes were isolated by pelleting the beads, and subjected to ECL-Western blotting using HA antibody. Results are representative of three (30 second exposure) or two (overnight exposure) independent experiments. C. Lysates of HEK-293T cells expressing SmgGDS-607-HA were prepared as increasing concentrations of total cellular protein (45 μg - 2250 μg) (left) or 1000 μg total cellular protein (right), and allowed to interact with the biotin-K-Ras CVIL peptide (10 μg) that was pre-bound to streptavidin beads. Full-length recombinant GST-K-Ras-CVIM or -CVIL proteins were added at increasing concentrations (0.1 μg - 1 μg) to the reaction mixtures (right). SmgGDS-607-HA that was bound to the biotin-K-Ras CVIL peptide was isolated by pelleting the streptavidin beads, and then subjected to ECL-Western blotting using HA antibody. Results are representative of two (left) or three (right) independent experiments. The graph shows the optical density (mean ± S.E.) of the immunoblotted SmgGDS-607-HA protein that was pulled down by the biotin-K-Ras CVIL peptide in the presence of the indicated concentrations of full-length recombinant GST-K-Ras-CVIM or -CVIL proteins. (ns, not significant; *, p<0.05 by One-Way ANOVA with Dunnett’s post-test compared to the control value from the sample that did not receive full-length recombinant GST-K-Ras protein.

**Figure 4:** SmgGDS-607 recognizes the last amino acid of Rap1B for binding. A. The C-terminal region of Rap1B is depicted in two segments: The PBR identified by the basic lysines and arginines, followed by the CAAX motif. WT Rap1B has a CAAX sequence of CQLL which allows for the protein to become geranylgeranylated. The last amino acid of Rap1B was mutated to a methionine for this study. B. HEK-293T cells were transfected with a cDNA encoding SmgGDS-607-HA, SmgGDS-558-HA, or the HA vector plus a cDNA encoding the Myc-tagged WT Rap1B CQLL or Rap1B CQLM mutant, or a Myc
vector. After 24 h, the cells were lysed and an aliquot of each lysate was subjected to ECL-Western blotting using Myc antibody (Cell Lysates). The remaining volume of each cell lysate was immunoprecipitated with HA antibody, followed by ECL-Western blotting using HA antibody and Myc antibody (HA Immunoprecipitates). Results are shown as the optical density of IP Myc-Rap1B divided by the optical density of IP SmgGDS-HA and are the mean ± S.E. of three independent experiments. (ns, not significant; **, p<0.01 by Student’s t test). A non-specific background band in the immunoblot is indicated by the asterisk (*).

Figure 5: SmgGDS-607 binds directly to both the prenylated and non-prenylated forms of a Rap1B C-terminal peptide, and SmgGDS-558 binds to the prenylated form of the peptide. A. The structure of the free thiol Rap1B peptide is shown, where the asterisk indicates the farnesyl (left), geranylgeranyl (middle), or C10-m-BP (right) group. The C10 group contains the photoactive benzophenone-containing residue which allows for photo-crosslinking to a substrate. B and C. The indicated Biotin-PEG-Rap1B peptides in the free thiol (F.T.), farnesylated (Far.), or geranylgeranylated (GGer.) forms were pre-bound to streptavidin beads and allowed to interact with lysates of HEK-293T cells expressing either SmgGDS-607-HA, SmgGDS-558-HA, or HA Vector. The bound complexes, whole cell lysates (B), and excess protein not bound to the streptavidin beads (C) were subjected to ECL-Western blotting using HA antibody. Results are representative of three independent experiments. D. Biotin-PEG-Rap1B C10-m-BP peptides were allowed to interact with HEK-293T cell lysates containing either SmgGDS-607-HA or HA vector and exposed to increasing time of UV light (1, 10, 30, and 60 min). The control sample was wrapped in tinfoil and exposed to UV light for 60 min. Streptavidin beads were used to pull down the complexes of SmgGDS-607-HA cross-linked to the biotinylated Rap1B peptide, and then the complexes and whole cell lysates were subjected to ECL-Western blotting using HA antibody. Results are representative of three independent experiments.

Figure 6: SmgGDS-608/607 and SmgGDS-559/558 share similar structural domains with differences in the N-terminal region. A. Pymol homology models of SmgGDS-608 and SmgGDS-559 are presented based on a previous published model of SmgGDS-608 (28). SmgGDS-608 corresponds to SmgGDS-607, whereas SmgGDS-559 corresponds to SmgGDS-558, as described in the Materials and Methods. The armadillo (ARM) domains in the proteins are designated A – M, with ARM C (yellow) being spliced out of SmgGDS-559/558. Both SmgGDS-608/607 and SmgGDS-559/558 have similar structures in ARMs D – M (green), but SmgGDS-608/607 differs significantly from SmgGDS-559/558 in the structure of ARMs A and B (blue). B. Our results support a model in which a GTPase ending with a methionine enters the farnesylation pathway without the aid of SmgGDS-607. The farnesylated GTPase then interacts with SmgGDS-558 and traffics to the plasma membrane. In contrast, a small GTPase ending with a leucine interacts with SmgGDS-607 prior to becoming prenylated by the geranylgeranyltransferase. The geranylgeranylated GTPase then interacts with SmgGDS-558 and traffics to the plasma membrane. A FTI promotes the alternate geranylgeranylation of a GTPase that is normally farnesylated (e.g. K-Ras) and this leads to an increased association between a GTPase ending in a methionine and SmgGDS-607. More analyses are needed to determine if the interaction of WT K-Ras with SmgGDS-607 is needed for the alternate geranylgeranylation of this GTPase in cells treated with FTI (red arrows).
### Table 1

#### SmgGDS-607

| Rank | Name of Enzyme Homolog                      | Cscore<sup>EC</sup> | PDB Hit | TM-score | Predicted Active Site Residues |
|------|---------------------------------------------|---------------------|---------|-----------|-------------------------------|
| 1    | Vacular ATP Synthase Subunit H              | 0.177               | 1ho8A   | 0.387     | NA                            |
| 2    | PME-1 and PP2A core enzyme                  | 0.157               | 3c5wA   | 0.279     | 372                           |
| 3    | Immunoglobulin A1 protease                  | 0.15                | 3h09B   | 0.411     | 364                           |
| 4    | 1,4-alpha-glucan-branching enzyme           | 0.136               | 3k1dA   | 0.388     | 335 384                      |
| 5    | Fatty Acid Synthase Subunit Alpha           | 0.133               | 2vkgG   | 0.362     | 225                           |

#### SmgGDS-558

| Rank | Name of Enzyme Homolog                      | Cscore<sup>EC</sup> | PDB Hit | TM-score | Predicted Active Site Residues |
|------|---------------------------------------------|---------------------|---------|-----------|-------------------------------|
| 1    | Vacular ATP Synthase Subunit H              | 0.25                | 1ho8A   | 0.427     | 282 284 289 291 334           |
| 2    | Protein farnesyltransferase                 | 0.191               | 3dpyA   | 0.38      | NA                            |
| 3    | PME-1 and PP2A core enzyme                  | 0.181               | 3c5wA   | 0.29      | 239 325                      |
| 4    | O-linked GlcIAC transferase                | 0.163               | 1w3bA   | 0.426     | 311 344                      |
| 5    | Immunoglobulin A1 protease                  | 0.162               | 3h09B   | 0.418     | 298 315                      |
SmgGDS-607 is a CAAX-specific binding protein

**Figure 1**

| GTPase | Polybasic region (PBR) | CAAX | (prenylation) |
|--------|------------------------|------|--------------|
| K-Ras: | K K K K K K S K T K | CV I M | (farnesylated) |
| K-Ras: | K K K K K K S K T K | CV I L | (geranyl/geranylated) |
| K-Ras: | K K K K K K S K T K | S V I M | (non-prenylated) |
| K-Ras: | K K K K K K S K T K | S V I L | (non-prenylated) |

**B**

HA-tagged SmgGDS:
- SmgGDS-607
- SmgGDS-558
- HA-Vector

**C**

HA-tagged SmgGDS:
- SmgGDS-607
- SmgGDS-558

FTI (10 μM)
SmgGDS-607 is a CAAX-specific binding protein

Figure 2

A

B

Endogenous SmgGDS
Gαi Subunit

myc-K-Ras
Non-Prenyl
Prenyl

Cell Lysates

Endogenous SmgGDS
Gαi Subunit

myc-K-Ras
Non-Prenyl
Prenyl

K-Ras CVIM
K-Ras CVIL

Total Cell Lysate

Cell Lysates

K-RAS CVIM
K-RAS CVIL

FTI
GTT
FTI+GTT
MEV
VEHICLE
A
D
A
D
A
D
A
D
A
D
A
D

64 kDa
-49 kDa
-37 kDa
-26 kDa

3.0
2.5
2.0
1.5
1.0
0.5
0.0

1 2 3 4 5 6 7 8 9 10

FTI
GTT
FTI+GTT
MEV
VEHICLE
A
D
A
D
A
D
A
D
A
D

64 kDa
-49 kDa
-37 kDa
-26 kDa

3.0
2.5
2.0
1.5
1.0
0.5
0.0

1 2 3 4 5 6 7 8 9 10

FTI
GTT
FTI+GTT
MEV
VEHICLE
A
D
A
D
A
D
A
D
A
D

64 kDa
-49 kDa
-37 kDa
-26 kDa

3.0
2.5
2.0
1.5
1.0
0.5
0.0

1 2 3 4 5 6 7 8 9 10

FTI
GTT
FTI+GTT
MEV
VEHICLE
A
D
A
D
A
D
A
D
A
D

64 kDa
-49 kDa
-37 kDa
-26 kDa

3.0
2.5
2.0
1.5
1.0
0.5
0.0

1 2 3 4 5 6 7 8 9 10

FTI
GTT
FTI+GTT
MEV
VEHICLE
A
D
A
D
A
D
A
D
A
D

64 kDa
-49 kDa
-37 kDa
-26 kDa

3.0
2.5
2.0
1.5
1.0
0.5
0.0

1 2 3 4 5 6 7 8 9 10

FTI
GTT
FTI+GTT
MEV
VEHICLE
A
D
A
D
A
D
A
D
A
D

64 kDa
-49 kDa
-37 kDa
-26 kDa

3.0
2.5
2.0
1.5
1.0
0.5
0.0

1 2 3 4 5 6 7 8 9 10

FTI
GTT
FTI+GTT
MEV
VEHICLE
A
D
A
D
A
D
A
D
A
D

64 kDa
-49 kDa
-37 kDa
-26 kDa

3.0
2.5
2.0
1.5
1.0
0.5
0.0

1 2 3 4 5 6 7 8 9 10
SmgGDS-607 is a CAAX-specific binding protein

Figure 3

A

B

HA-tagged SmgGDS:

HA-tagged SmgGDS

HA-tagged SmgGDS

Protein not bound to Strept. beads WCL

WCL

Biotin-K-Ras CVIL Peptide

SmgGDS-607-HA

HEK Cell Lysate (µg)

1 2 3 4 5

Streptavidin IP

SmgGDS-607-HA

Full Length Recombinant

GSt-K-Ras CAAX (µg)

0 0.1 µg 0.5 µg 1 µg

Streptavidin IP

Optical Density

0 0.4 0.8 1.2

GST-Myc-K-Ras CVIM GST-Myc-K-Ras CVIL

n=3
SmGDS-607 is a CAAX-specific binding protein

Figure 4

A

| GTPase   | Polybasic region (PBR) | CAAX (prenylation) |
|----------|------------------------|--------------------|
| Rap1B    | VPGBKKS              | CQLL (geranylgeranylated) |
| Rap1B    | VPGBKKS              | CQLM (farnesylated)  |

B

HA-tagged SmgGDS:  
myc-Rap1B-CAAX

|            | SmgGDS-607 | SmgGDS-558 |
|------------|------------|------------|
| myc-Rap1B  | CQLL       | CQLM       |
| Vector     | CQLL       | CQLM       |

HA Immunoprecipitates

Cell Lysates

Optical Density

|          | SmgGDS-607 | SmgGDS-558 |
|----------|------------|------------|
| CQLL     | ns         |            |
| CQLM     |            |            |

n=3
SmgGDS-607 is a CAAX-specific binding protein

Figure 5

A

Biotin-PEG<sub>20</sub>-V P G K A R K S S C Q L L (F.T.)

\[
\text{Farnesyl diphosphate (Far.)}
\]

\[
\text{Geranylgeranyl diphosphate (GGer.)}
\]

\[
\text{C10-m-BP diphosphate (C10)}
\]

B

| Biotin-Peptide: | Rap1B Free Thiol | Rap1B Ger.geranyl |
|----------------|------------------|-------------------|
| SmgGDS-HA:     |                  |                   |
| 1               | 2                | 3                 |
| 4               | 5                | 6                 |
| Streptavidin Immunoprecipitation |
| SmgGDS-HA:     |                  |                   |
| 1               | 2                | 3                 |
| 4               | 5                | 6                 |
| Whole Cell Lysates |

C

| Biotin Rap1B Peptide: | HA Vector | Sg 607-HA | Sg 555-HA |
|-----------------------|-----------|-----------|-----------|
| HA Vector             | F.T.      | F.T.      | F.T.      |
| Sg 607-HA             | F.T.      | F.T.      | F.T.      |
| Sg 555-HA             | F.T.      | F.T.      | F.T.      |
| SmgGDS-HA:            | 1         | 2         | 3         |
| 4                     | 5         | 6         | 7         |
| 8                     | 9         | 10        | 11        |
| 12                    |           |           |           |
| Streptavidin Immunoprecipitation |
| SmgGDS-HA:            | 1         | 2         | 3         |
| 4                     | 5         | 6         | 7         |
| 8                     | 9         | 10        | 11        |
| 12                    |           |           |           |
| Excess Protein Not Bound to Streptavidin Beads |

D

| Ultraviolet Light Exposure (t) | SmgGDS 607-HA | Whole Cell Lysate |
|-------------------------------|---------------|-------------------|
| 1 min                         |               |                   |
| 10 min                        |               |                   |
| 30 min                        |               |                   |
| 60 min                        |               |                   |
| (Control)                     |               |                   |
| HA Vector                     | F.T.          | F.T.              |
| Sg 607-HA                     | F.T.          | F.T.              |
| Sg 555-HA                     | F.T.          | F.T.              |
| SmgGDS                        | 1             | 2                  |
| 3                             | 4             | 5                  |
| 6                             | 7             | 8                  |
| Streptavidin Immunoprecipitation |

Graphical representation of Optical Density:

- SmgGDS-607
- SmgGDS-558
Figure 6

A

SmgGDS-608

B

SmgGDS-559

FTase

FTI

GGTase

GTPase-CAAL

GTPase-CAAM

Farnesylation Pathway

Geranylgeranylation Pathway

Newly Synthesized GTPase

SmgGDS-607 is a CAAX-specific binding protein
The chaperone protein SmgGDS interacts with small GTPases entering the prenylation pathway by recognizing the last amino acid in the CAAX motif

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