The chaperone SmgGDS-607 has a dual role, both activating and inhibiting farnesylation of small GTPases

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Ras family small GTPases undergo prenylation (such as farnesylation) for proper localization to the plasma membrane, where they can initiate oncogenic signaling pathways. Small GTP-binding protein GDP-dissociation stimulator (SmgGDS) proteins are chaperones that bind and traffic small GTPases, although their exact cellular function is unknown. Initially, SmgGDS proteins were classified as guanine nucleotide exchange factors, but recent findings suggest that SmgGDS proteins also regulate prenylation of small GTPases in vivo in a substrate-selective manner. SmgGDS-607 recognizes the polybasic region and the CAAX box of several small GTPases and inhibits prenylation by impeding their entry into the geranylgeranylation pathway. Here, using recombinant and purified enzymes for prenylation and protein-binding assays, we demonstrate that SmgGDS-607 differentially regulates farnesylation of several small GTPases. SmgGDS-607 inhibited farnesylation of some proteins, such as DiRas1, by sequestering the protein and limiting modification catalyzed by protein farnesyltransferase (FTase). We found that the competitive binding affinities of the small GTPase for SmgGDS-607 and FTase dictate the extent of this inhibition. Additionally, we discovered that SmgGDS-607 increases the rate of farnesylation of HRas by enhancing product release from FTase. Our work indicates that SmgGDS-607 binds to a broad range of small GTPases and does not require a PBR for recognition. Together, these results provide mechanistic insight into SmgGDS-607-mediated regulation of farnesylation of small GTPases and suggest that SmgGDS-607 has multiple modes of substrate recognition.

Several small GTPases, including members of the Ras, Rho, and Rap families, are post-translationally modified by protein prenylation (1–4). Protein prenylation consists of the addition of an isoprenoid lipid to the cysteine residue within the C-terminal consensus sequence known as the CAAX box, where C is the cysteine to be modified, A is generally an aliphatic residue, and the X residue is variable. The prenylation process includes three main steps: prenylation, proteolysis, and methylation. The first step, prenylation, is catalyzed by protein farnesyltransferase (FTase)2 and protein geranylgeranyltransferase-I (GGTase-I) to covalently attach a 15-carbon farnesyl or a 20-carbon geranylgeranyl moiety, respectively, to the cysteine thiol (5). Following lipid attachment, a prenyl protein protease in the endoplasmic reticulum membrane, RAS-converting CAAX endopeptidase 1 (Rce1), catalyzes cleavage of the last three residues (-AAAX) of small GTPase CAAX proteins. Finally, in step three, isoprenylcysteine carboxymethyltransferase (ICMT) catalyzes the addition of a methyl group to the newly exposed C-terminus, making the C-terminus more hydrophobic (5, 6). Depending on the small GTPase identity, after the third step, the protein may undergo additional modification steps before trafficking to the plasma membrane. Once anchored in the plasma membrane, small GTPases fulfill their biological role, operating as molecular switches shifting between a GDP-bound inactive and a GTP-bound active state, where they interact with one or more downstream effector proteins initiating cell signaling pathways. Hence, CAAX prenylation plays a crucial role in small GTPase function (3, 7).

For over 30 years, Ras proteins (KRas4A, KRas4B, NRas, and HRas) have attracted attention because of their connection to human cancer. Oncogenic Ras mutations, of which the most prominent occur at Gly-12, Gly-13, and Gln-61, can reduce or eliminate the inherent GTPase activity, leading to constitutive GTP binding and therefore activation of signaling pathways (8–10). Such Ras mutants are not inactivated by normal cellular mechanisms, and unchecked activity is associated with human tumor pathogenesis. KRas is the isoform that is mutated most often (85%) in cancers with a Ras missense mutation, followed by NRas (12%) and HRas (3%) (11). One of many efforts to control aberrant GTase signaling focuses on impeding Ras localization to the plasma membrane by targeting FTase prenylation through small-molecule inhibitors (8). One caveat of FTase inhibitors (FTIs) is their inability to block prenylation of

2 The abbreviations used are: FTase, protein farnesyltransferase; PBR, polybasic region; GGTase-I, geranylgeranyltransferase type I; FTI, farnesyltransferase inhibitor; SmgGDS, small GTP-binding protein GDP-disociation stimulator protein; PPP, farnesyl diphosphate; IPTG, isopropyl β–D–1-thiogalactopyranoside; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; Mocco, molecular mass cutoff; BLU, biolayer interferometry; N-NTA, nickel–nitrilotriacetic acid; Bisic, N-V-bis(2-hydroxyethyl)glycine; WT, wild-type; HEPPSO, N-(2-hydroxyethyl)piperazine-N′-(2-hydroxypropanesulfonic acid).

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1 This article contains Table S1 and Figs. S1 and S2.

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**Effects of SmgGDS-607 on small GTPase farnesylation**

KRAs and NRAs. In the presence of FTIs, GGTase-I catalyzes geranylgeranylation of KRAs and NRAs, which allows for normal function in the cell (12–14). The cross-reactivity of these proteins with both prenyltransferases is related to the sequence of the CAAX box, which includes a methionine at the X position (-CAAM) (15). Combination treatment with both FTIs and GGTase inhibitors has been shown to block prenylation of KRAs and NRAs in mice, but only at lethally high doses (16, 17). Thus, a novel strategy that can prevent membrane association of oncogenic Ras proteins might aid in cancer treatment.

CAAX-protein prenylation has been generally assumed to be unregulated in the cell (18). However, recent studies indicate that splice variants of SmgGDS (small GTP-binding protein GDP-dissociation stimulator) proteins bind small GTPases and regulate their entry into the prenylation pathway (19, 20). There are two splice variants of SmgGDS, SmgGDS-607 and SmgGDS-558, and both bind multiple Ras and Rho family members by recognizing their C-terminal PBRs and CAAX boxes (19–22). Previous data suggest that SmgGDS-607 binds newly synthesized, nonprenylated GTPases, whereas SmgGDS-558 binds prenylated GTPases, potentially helping them traffic to the plasma membrane (19). Recent in vitro studies show that SmgGDS-607 inhibits the geranylgeranylation of RhoA in a nucleotide-dependent manner, and this inhibition occurs through RhoA substrate sequestration rather than inhibition of GGTase-I (20). Because SmgGDS-607 binds nonprenylated GTPases and inhibits prenylation, this protein is proposed to function as a gatekeeper by regulating small GTPase entry into the prenylation pathway.

Although the role of SmgGDS-607 in inhibiting geranylgeranylation is well-established (20), the role of this protein in regulating the farnesylation pathway is still not known. So far, five small GTPases that go through the farnesylation pathway (KRas4B, HRas, NRas, DiRas1, and DiRas2) have been demonstrated to associate with SmgGDS-607 in cells (21–24). Pull-down experiments have demonstrated that SmgGDS-607 associates with WT, constitutively active (G12V), and dominant negative (S17N) KRas4B and that this association appears to be mediated by the PBR (19, 21). Additionally, more KRas4B pulls down with SmgGDS-607 when cells are treated with an FTI, compared with cells that are untreated (22). To further characterize the role of SmgGDS-607 in regulating farnesylation, we assayed the effects of SmgGDS-607 on in vitro prenylation of three representative FTase substrates: KRas4B, HRas, and DiRas1. These three substrates capture the different types of C-terminal tails in the FTase substrates recognized by SmgGDS-607. Furthermore, we measured the binding affinities of Ras proteins for the prenyltransferases and SmgGDS-607. These data demonstrate that SmgGDS-607 does not significantly affect the rate of farnesylation of KRas4B while inhibiting farnesylation of DiRas1, a Ras-like protein homologous to KRas4B. These differential effects are explained by the relative binding affinities of the small GTPase for SmgGDS-607 and FTase. Surprisingly, SmgGDS-607 enhances the rate of farnesylation of HRas, an effect that has never been seen before with any other small GTPase, by increasing the rate of product dissociation from FTase. These results elucidate a novel cellular mechanism for regulation of protein farnesylation. This mechanism suggests that SmgGDS-607 enhances entry of certain small GTPases into the farnesylation pathway, implying that small molecules targeting the SmgGDS-607 and Ras interaction may have therapeutic value.

### Results

**SmgGDS-607 blocks farnesylation of DiRas1 but not KRas4B**

SmgGDS-607 inhibits geranylgeranylation of RhoA catalyzed by GGTase-I by binding to and blocking the prenylation site (20). Furthermore, SmgGDS-607 was previously demonstrated to pulldown with small GTPases ending in methionine, such as KRas4B, only in the presence of an FTI that leads to alternative geranylgeranylation (22). However, SmgGDS-607 has not been demonstrated to regulate farnesylation of small GTPases. To test SmgGDS-607-mediated regulation, we used a radiolabel-incorporation assay to monitor levels of farnesylation of small GTPases, specifically KRas4B and DiRas1, in the presence of SmgGDS-607. Although DiRas1 and KRas4B belong to different branches of the Ras family, they share 36% sequence identity, end in methionine (Table 1), and are modified by FTase (25). Additionally, Bergom et al. (23) demonstrated that DiRas1 antagonizes the interaction of SmgGDS-558 with KRas4B.

Under in vitro conditions, the addition of increasing concentrations of SmgGDS-607 minimally inhibits the extent of farnesylation of KRas4B, with 32% inhibition at 20 μM SmgGDS-607 after 5 min of incubation. In contrast, the addition of SmgGDS-607 significantly decreases farnesyl incorporation in DiRas1, with more than 90% inhibition at 5 μM SmgGDS-607 (Fig. 1A) as compared with ~10% inhibition for KRas4B under similar conditions. Together, these data demonstrate that SmgGDS-607 can directly block farnesylation of an FTase substrate that ends in methionine and does not require the presence of an FTI.

**SmgGDS-607 does not inhibit FTase reactivity**

To investigate whether the observed inhibition of farnesylation by SmgGDS-607 is due to SmgGDS-607 directly inhibiting FTase reactivity, we tested the effect of SmgGDS-607 on the farnesylation rate of a dansylated peptide substrate (dansyl-TKCVIM) that mimics the CAAX box of KRas4B and has high affinity for FTase (0.86 ± 0.08 nM) (27). The addition of 5 μM SmgGDS-607 did not significantly affect FTase activity (Fig. 1B). Both kinetic parameters, $k_{cat}$ and $V_{max}$, were unchanged by the presence of SmgGDS-607 (Table 2). These data demonstrate that SmgGDS-607 does not directly inhibit FTase. Rather, the observed inhibition (Fig. 1A) occurs by competition for binding the FTase substrate.

### Table 1

| Small GTPase | Polybasic region | CAAX box | No. of amino acids in sequence |
|-------------|-----------------|----------|-------------------------------|
| KRas4B      | CKKICG       | CVM      | 188                           |
| KRas4B M188L| CKKICG       | CVIL     | 188                           |
| DiRas1      | CVKRTTRK     | CTLM     | 198                           |
| HRas*       | PDEGSPCQSCK  | CVLS     | 189                           |

* Contrary to the other GTPases tested, HRas does not contain a recognizable polybasic region.

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in the presence of SmgGDS-607. A, SmgGDS-607 blocks the extent of farnesylation of DiRas1, but not KRas4B, in a dose-dependent manner. Both KRas4B and DiRas1 end with a methionine in their CAAX box, consistent with the observed farnesylation catalyzed by FTase. KRas4B or DiRas1 (2.5 μM final) was incubated with GDP (25 μM), FTase (25 nm), [3H]FPP (4 μM), and eight different concentrations of SmgGDS-607. The reactions were stopped after 5 min (KRas4B) or 6 min (DiRas1), and incorporation of radiolabeled farnesyl was measured as described under “Experimental procedures.” The data points are from single reactions at each SmgGDS-607 concentration. The results are representative of three independent experiments. *, *p < 0.05 by one-way analysis of variance with Dunnett’s post-test compared with control value that was not incubated with SmgGDS-607. The data points are from single reactions at each SmgGDS-607 concentration. The results are representative of three independent experiments.

Table 2

| [SmgGDS-607] (μM) | kcat (s⁻¹) | Km (μM) |
|------------------|-----------|---------|
| 5 μM             | 0.08 ± 0.01 | 3 ± 1   |

Differential binding affinities for SmgGDS-607 with FTase substrates

To determine a possible mechanism to explain the differential effects of SmgGDS-607 on blocking farnesylation of DiRas1 compared with KRas4B, we measured the affinities of SmgGDS-607 for several small GTPases using biolayer interferometry (BLI, OctetRed96) with biotinylated small GTPases and varying concentrations of SmgGDS-607 (26). BLI data for SmgGDS-607 binding to KRas4B, including the kinetic association rate constant (kcat), the kinetic dissociation rate constants (koff), and the equilibrium end point are shown in Fig. 2. The dissociation constants (Km) for KRas4B, HRas, and DiRas1, and HRas were determined from the fit of a single binding isotherm to the end-point data (Fig. 2, B and C). Comparable binding data for all three small GTPases are summarized in Table 3 and Table S1.

An electronegative patch in the structure of SmgGDS-607 is predicted to be pivotal for interactions with the positive charges in the PBR of small GTPases (19, 23). In the KRas4B PBR, tandem repeats of lysine residues provide a highly positively charged region that could interact with the electronegative patch in SmgGDS-607, as compared with the more dispersed positive charges in the PBR of DiRas1 (Table 1). Based on this, we predicted that KRas4B would bind to SmgGDS-607 with high affinity. However, the affinity of KRas4B for SmgGDS-607 is 1.5 ± 0.4 μM, which is >300-fold weaker than the DiRas1 affinity at 4.7 ± 0.9 nM, suggesting a more complicated model of substrate recognition.

Previous data indicated that SmgGDS-607 pulls down higher levels of KRas4B when the terminal amino acid is altered to leucine from methionine (KRas4B M188L) (22). To investigate this phenomenon, we repeated the binding assay using KRas4B M188L, demonstrating a 13-fold increased affinity for SmgGDS-607 (Table 3). We next measured the affinity of SmgGDS-607 for HRas, a small GTPase that does not contain a canonical PBR and ends with a different CAAX (−CVLS) sequence. The affinity of HRas for SmgGDS-607 is >9 μM, which is >6-fold weaker than KRas4B and >1,000-fold weaker than DiRas1, indicating that HRas has the weakest binding affinity out of all the substrates tested. The contrasting affinity of HRas compared with KRas4B indicates that the PBR might regulate the affinity of the interaction with SmgGDS-607. Furthermore, these data suggest that the PBR motif in KRas4B may not optimally interact with SmgGDS-607 because the affinity is weaker than DiRas1. The enhanced binding affinity for the KRas4B M188L mutant supports the proposal that SmgGDS-607 recognizes the last residue in KRas4B and that the amino acid sequence of the CAAX box is an important determinant of binding affinity and substrate selectivity.

FTase binds to small GTPases with differential binding affinities

Measurement of direct binding affinities between FTase and full-length small GTPases has proven a challenge previously. To solve this issue we have used BLI to measure, for the first time, direct real-time binding of FTase to KRas4B and DiRas1 to determine dissociation constants, Kd, for these full-length proteins. Previously, affinities for rat FTase binding CAAX peptides with varied terminal residues were determined; a peptide corresponding to the C-terminus of KRas4B (TKCVIM) has a Kd of 0.86 ± 0.08 nm (27). Using BLI, we measured the affinity of human FTase for KRas4B as Kd = 2.2 ± 0.7 nm (Table 3), comparable with the value measured for the peptide. Interestingly, when the native CAAX sequence CVIM is replaced with
CVIL, the affinity for human FTase decreases by 500-fold ($K_D = 1.1 \pm 0.3 \mu M$). This result was unexpected because for peptides there is minimal difference in the rat FTase dissociation constants for TKCVIL compared with TKCVIM (1.1 \pm 0.1 and 0.86 \pm 0.08 nM, respectively) (27). These data indicate that the change in affinity for the full-length protein is not determined solely by an altered interaction of FTase with the CAAX tail. For full-length DiRas1 (CAAX = CTLM), the measured dissociation constant for human FTase is 40 \pm 2 nM (Table 3) which is 8-fold weaker than the measured $K_D$ for rat FTase with the TKCTL peptide (5.2 \pm 0.8 nM) (28). These data also suggest that other factors, such as interaction with the PBR region and/or steric hindrance of the C-terminal tail by the full-length protein, explain this weaker affinity.

**SmgGDS-607 blocks farnesylation of KRas4B when the CAAX ends in leucine**

To further test whether the ability of SmgGDS-607 to block farnesylation of small GTPases depends on the relative affinities of FTase and SmgGDS-607, we measured whether SmgGDS-607 blocks farnesylation of mutant KRas4B M188L. The results are representative of three independent experiments.

Table 3

Summary of thermodynamic values for SmgGDS-607 or FTase binding to different small GTPases

The values were measured by biolayer interferometry as described under “Experimental procedures” and the in legend of Fig. 2. $K_D$, was determined from a hyperbolic equation fit to the end-point data from the BLI response obtained during the association step between the small GTPases and SmgGDS-607 or FTase. The errors in the $K_D$ values are from the standard error of the fit of the binding isotherm to the data. The uncertainty in the ratio was calculated by error propagation (39).

| Binding to SmgGDS-607 | CAAX identity | $K_D$ (nM) | $K_D$ ($K_D^{FTase}$) |
|-----------------------|---------------|------------|----------------------|
| KRas4B                | CVIM          | 1500 ± 370 | 700 ± 300            |
| KRas4B M188L          | CVIL          | 110 ± 20   | 0.10 ± 0.04          |
| DiRas1                | CTLM          | 4.7 ± 0.9  | 0.12 ± 0.02          |
| HRas                  | CVLS          | >9000      |                      |

| Binding to FTase      | CAAX identity | $K_D$ (nM) | $K_D$ ($K_D^{FTase}$) |
|-----------------------|---------------|------------|----------------------|
| KRas4B                | CVIM          | 2.2 ± 0.7  |                      |
| KRas4B M188L          | CVIL          | 1100 ± 370 |                      |
| DiRas1                | CTLM          | 40 ± 2     |                      |
| HRas                  | CVLS          | ND*        |                      |

*Affinity of HRas for FTase was not determined (ND)*

Figure 3. SmgGDS-607 blocks farnesylation of mutant KRas4B M188L, a less reactive FTase substrate. Radiolabel prenylation assay was performed similarly to Fig. 1A. KRas4B M188L (2.5 \mu M final) was incubated with GDP (25 \mu M), FTase (25 \mu M), [3H]FPP (4 \mu M), and eight different concentrations of SmgGDS-607. The reactions were stopped after 30 min of incubation. The dotted line represents the effects of SmgGDS-607 on KRas4B farnesylation from Fig. 1A. Data points are from single reactions at each SmgGDS-607 concentration. The results are representative of three independent experiments.
crease in the $K_d^{FTase}/K_d^{SmgGDS-607}$ for the mutant compared with WT of $>5,000$-fold. Consistent with this, $2.5 \mu M$ SmgGDS-607 inhibits $>90\%$ of the farnesylation of KRas4B M188L compared with $\sim 10\%$ of WT KRas4B under comparable conditions. Contrary to KRas4B, for KRas4B M188L, the reactions were allowed to proceed for 30 min because farnesylation of this mutant is slower. At 30 min, the amount of product formed is comparable with that of KRas4B after a 5-min incubation. This result provides additional evidence that SmgGDS-607 regulates farnesylation of KRas4B, and likely other small GTPases, is driven by differences in binding affinities for FTase and SmgGDS-607. To further examine this mechanism, we simulated the SmgGDS-607 concentration dependence of inhibition of GTPase farnesylation using the KinTek Explorer chemical kinetics software with a differential binding model (Fig. S1). Our modeling provides qualitative dose-response curves consistent with those obtained empirically (Figs. 1 and 3), confirming that SmgGDS-mediated regulation for KRas4B and DiRas1 can be explained by a differential binding model. Taken together, these data demonstrate that regulation of farnesylation by SmgGDS-607 is driven by the differential binding affinities, suggesting that in a cellular context SmgGDS-607 competes with FTase for binding of small GTPases.

SmgGDS-607 enhances farnesylation of HRas

To date, there has been no evidence that SmgGDS-607 regulates prenylation of small GTPases without a PBR sequence. To examine this, we measured the effect of SmgGDS-607 on prenylation of HRas which contains no identified PBR and has a CAXX sequence that ends in serine (CVLS). Previous pulldown data suggested that SmgGDS-607 interacts with both dominant-negative and nucleotide-free forms of HRas (21). However, no one has examined the effects of SmgGDS-607 on farnesylation of HRas. Unexpectedly, the addition of $10 \mu M$ SmgGDS-607 enhanced farnesylation of HRas by $\sim 5$-fold, while having almost no effect on geranylgeranylation (Fig. 4A). Minimal modification (<1%) was observed after a 20-min incubation of 2.5 $\mu M$ HRas with 1 $\mu M$ GGTase-I; the addition of 10 $\mu M$ SmgGDS-607 did not increase geranylgeranylation. To better understand this farnesylation enhancement, we measured the effect of SmgGDS-607 on the rate of steady-state turnover (Fig. 4B). Increasing concentrations of SmgGDS-607 enhance the rate constant for farnesylation of 2.5 $\mu M$ HRas by 4.4-fold (Fig. 4A), with a $k_{max}$ of 0.00071 ± 0.00009 s$^{-1}$ and a $K_{1/2}$ of 1.4 ± 0.7 $\mu M$.

The enhancement of HRas farnesylation by SmgGDS-607 could be due to either or both: 1) decreasing the $K_m$ for HRas and/or 2) increasing turnover ($k_{cat}$) of farnesylated HRas catalyzed by FTase. To distinguish between these alternatives, we measured the effect of SmgGDS-607 on the multiple turnover kinetic parameters $k_{cat}$ and $K_m$ for FTase-catalyzed modification of HRas using the radiolabel-incorporation assay with HRas concentrations that were at least two times below and above the $K_m$ (Fig. 5A). These kinetic data reveal that SmgGDS-607 has a minimal effect on the value of $K_m$ while increasing the values of $k_{cat}$ and $k_{cat}/K_m$ by $>5$-fold (Table 4). For peptide substrates, the farnesylation step is rapid, and product release is rate-limiting for turnover (29, 30); however, this has not been evaluated for protein substrates. To test whether the effect of SmgGDS-607 on $k_{cat}$ is due to enhancement of product release or chemistry, we measured farnesylation of HRas under single-turnover conditions, where FTase and HRas concentrations are in excess of the FPP concentration. Under these conditions, the observed rate constant, $k_{obs}$, for farnesylation of HRas is 0.0021 ± 0.0002 s$^{-1}$, which is 16-fold faster than the value of $k_{cat}$ indicating that a step after farnesylation, such as product release, is the rate-limiting step for turnover. The addition of $10 \mu M$ SmgGDS-607 increases the observed single turnover rate constant for farnesylation of HRas by 3-fold, but this rate remains 4-fold faster than the multiple-turnover rate constant (Fig. 5B and Table 4). These data indicate that SmgGDS-607 has a modest effect on binding and/or catalysis while also increas-
Figure 5. SmgGDS-607 enhances HRas farnesylation by stimulating both chemistry and product release. A. Concentration dependence of farnesylation of HRas catalyzed by FTase in the presence of SmgGDS-607. The initial velocity for the reaction of 50 nM FTase, eight different concentrations of HRas, 25 μM GDP, [3H]FPP at twice the concentration of HRas, and either 0 or 10 μM SmgGDS-607 was measured from the time dependent change in incorporation of the radiolabel into HRas, as described under “Experimental procedures.” The initial rate of farnesylation (V/E) and standard error were determined by fitting a line (GraphPad Prism) to eight measurements of product formation at different times. The Michaelis–Menten equation was fit to these data to determine values for k_{cat}, K_m, k_{cat}/K_m, and standard errors, listed in Table 4. B. Single-turnover kinetics for farnesylation of HRas in the absence and presence of 10 μM SmgGDS-607. Under these conditions, FTase (500 nM) reacts with 2.5 μM HRas, 25 μM GDP, and limiting [3H]FPP (250 nM). For each condition, data points depict the amount of farnesylated HRas at seven different time points in a single reaction. The farnesylation rate constant k_{obs} and standard error were calculated by a fit (GraphPad Prism) of Equation 2 to the data as 0.0021 ± 0.0002 and 0.007 ± 0.001 s⁻¹ for without and with SmgGDS-607, respectively.

Table 4 Kinetic parameters for farnesylation of HRas catalyzed by FTase

| [SmgGDS-607] | k_{cat} | k_{m} | k_{cat}/k_{m} | k_{obs} |
|--------------|---------|-------|---------------|---------|
| —            | 0.00013 ± 0.00002 | 1.9 ± 0.8 | 68 | 0.0021 ± 0.0002 |
| 10 μM        | 0.0019 ± 0.0004 | 5 ± 2 | 380 | 0.007 ± 0.001 |

k_{obs} is the single-turnover rate constant for production of farnesylated HRas under limiting FPP conditions.

Discussion

Recent studies suggest that SmgGDS-607 can regulate geranylgeranylation of small GTPases by binding to and sequestering the nonprenylated form of the GTPase to inhibit prenylation (19, 22). Until now, a similar regulatory mechanism for farnesylation has not been observed. Our studies demonstrate that SmgGDS-607 can regulate farnesylation of FTase substrates by blocking the small GTPase from interacting with FTase. This mode of regulation is dictated by the differential binding affinities of the small GTPases between FTase and SmgGDS-607. Furthermore, unexpectedly our studies demonstrate that SmgGDS-607 increases farnesylation of HRas (and likely other small GTPases) by enhancing both catalysis and product release. These findings provide new insight into the regulation of small GTPases before and during the farnesylation pathway.

The structural architecture for small GTPases is defined by a globular body and a floppy tail that contains the PBR domain and the CAAX box. The tail, in particular, has been demonstrated to be crucial for recognition by the prenyltransferases (31–35). On the other hand, cellular data implicate both the tail and the body in SmgGDS recognition (21). Although pull-down experiments have been used to determine the relative affinity of SmgGDS-607 with small GTPases, the thermodynamic measurements for SmgGDS-607 and small GTPases that undergo farnesylation have not been reported. Previously, binding studies for SmgGDS-607 have been performed with RhoA, a GTase-I substrate (20, 35). Here, we sought to identify the determinants of SmgGDS-607 recognition of FTase substrates by assaying three different GTPases, KRas4B, DiRas1, and HRas. Our in vitro experiments demonstrate that the last residue in the CAAX box of KRas4B affects the binding affinity for SmgGDS-607, confirming cell-based work indicating the importance of this residue for selectivity (22). In cell experiments, WT KRas4B did not pull down with SmgGDS-607. However, when the C-terminal methionine was mutated to leucine, SmgGDS-607 co-immunoprecipitated with unprenylated KRas4B (22). Our data corroborate this finding because SmgGDS-607 binds the M188L mutant >300-fold more tightly than WT KRas4B, thus demonstrating that the CAAX box sequence is important for recognition. Additionally, our data suggest that other factors also regulate binding affinity, as indicated by the enhanced affinity of DiRas1 compared with KRas4B (Table 3). Recent studies suggest that the negatively charged region in SmgGDS-607 interacts with the PBR-CAAX motif of small GTPases (35, 36). The sequence of the PBR and the length of the tail are factors that could affect the direct contact with SmgGDS and/or the conformation of such GTPases, altering interaction with SmgGDS-607. Although both GTPases end in methionine, KRas4B and DiRas1 differ in their PBR sequence and tail length. The PBR domain of KRas4B contains tandem lysine repeats, which might not be optimal for binding to the dispersed electrongative region in SmgGDS-607, whereas the positive charges in the PBR domain of DiRas1 are more dispersed and include arginine residues (Table 1). Additionally, DiRas1 has a longer tail, which might provide optimal distal contacts with SmgGDS-607. In addition to the different features found on the tail of small GTPases, studies have shown that the bound nucleotide status of RhoA significantly influences the affinity for SmgGDS-607 (19, 20, 35, 43). In contrast, the affinity of SmgGDS-607 for DiRas1 increases...
modestly (2-fold) when the bound nucleotide is GTP compared with GDP (data not shown). These data suggest that the factors dictating binding affinity with SmgGDS-607 are complex and specific to each small GTPase.

Our binding studies demonstrate that the sequence of the C-terminal tails of small GTPases leads to differential affinities for SmgGDS-607 and FTase. Our model indicates that although SmgGDS-607 can bind to GTPases that undergo either farnesylation or geranylgeranylation, the regulation of prenylation is dictated by the relative binding affinities of each GTPase for SmgGDS-607 and the prenyltransferase (Fig. 6). For DiRas1 and KRas4B M188L, SmgGDS-607 inhibits farnesylation because of the higher affinity of the GTPase for SmgGDS-607 compared with FTase. However, SmgGDS-607 does not effectively inhibit farnesylation of KRas4B because of a combination of decreased affinity for SmgGDS-607 and increased affinity for FTase (Table 3). In contrast, geranylgeranylation of KRas4B is inhibited by SmgGDS-607 because of the enhanced affinity for SmgGDS-607 relative to GGTase-I (Table S1 and Fig. S2). Consistent with this model, previous data demonstrate that treatment of cells with a FTI increases the co-immunoprecipitation of SmgGDS-607 and KRas4B (22). Under these conditions, KRas4B can be alternatively geranylgeranylated. Because the binding affinity of KRas4B for GGTase-I is weaker than for SmgGDS-607 (Table S1), KRas4B preferentially binds to SmgGDS-607, and geranylgeranylation is inhibited. It is important to note that these events happen in a cellular environment where other small GTPases compete with KRas4B for binding to the prenyltransferases and SmgGDS-607. Particularly, cell-based competition experiments with DiRas1 demonstrate that DiRas1 decreases the amount of KRas4B that immunoprecipitates with SmgGDS-558 (23). Similarly, our binding data show that DiRas1 binds SmgGDS-607 >300-fold tighter than KRas4B, which predicts that increased unfarnesylated DiRas1 concentrations should antagonize SmgGDS-607 binding KRas4B in cells. Therefore, the prenylation pathways are constantly regulated by the levels of small GTPases and other binding partners. Furthermore, the PBR and CAAX sequences of a given GTPase are optimized for this regulatory function in the cell. Alteration of the association between small GTPases, such as KRas4B, and SmgGDS-607 by small molecules could be a novel target for effective therapeutics against small GTPase-associated cancers.

The data presented in this study, together with previous data (20, 22), show that SmgGDS-607 functions as a regulator of small GTPase geranylgeranylation by binding to nonprenylated small GTPases and blocking prenylation. Originally, SmgGDS was reported as a guanine nucleotide exchange factor for multiple small GTPases that contain a PBR. However, recently,
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SmgGDS was demonstrated to function as a guanine nucleotide exchange factor for only select GTPases, RhoA and RhoC (37). Other studies have reported that the PBR domain of small GTPases plays an important role for both nucleotide exchange and affinity for SmgGDS-607 (21, 38). Until now, no one has determined whether SmgGDS-607 also regulates small GTPases without a PBR, such as HRas. In this study, we demonstrate that SmgGDS-607 also enhances farnesylation of a noncanonical SmgGDS substrate, HRas. Our in vitro data support a model in which SmgGDS-607 forms a complex with both HRas and FTase that enhances both farnesylation and product release (Fig. 6B). Previous kinetic studies have demonstrated that the product dissociation is the rate-determining step in FTase turnover of peptides (29, 30). Furthermore, an additional FPP molecule binds to FTase to enhance dissociation of the prenylated peptide by trapping the farnesyl moiety in an alternate conformation in the “exit groove” (40). We hypothesize that SmgGDS-607 could enhance product release in a similar way. By interacting with both HRas and FTase, SmgGDS-607 could stabilize the alternate product conformation, which allows for enhanced turnover. Contrary to the other FTase substrates tested in this study, HRas does not contain a PBR on its tail and has modest affinity for SmgGDS-607 alone. Enhancement of prenylation has not been observed with GTPases that contain a PBR. Additionally, our kinetic study with dansylated peptide indicates that SmgGDS-607 does not directly interact with FTase. Hence, it suggests that the formation of a ternary FTase–protein–SmgGDS-607 complex might be specific for GTPases lacking a PBR.

Furthermore, the differential effects of SmgGDS-607 on farnesylation of small GTPases intensify the observed effects for a given substrate. Because FTase has multiple substrates in the cell, the specificity constant (kcat/Km) is the best index to compare the preference of FTase for reacting with different substrates. At 10 μM SmgGDS-607, the values of kcat/Km for farnesylation of small GTPases are affected as follows: DiRas1 decreases ≥10-fold, KRas4B remains essentially unchanged, and HRas increases 6-fold. Therefore, the ratio of kcat/Km values for HRas compared with DiRas1 changes by a factor of ≥60-fold. This calculation illustrates how the presence of SmgGDS-607 significantly alters the dynamics and levels of Ras farnesylation in the cell.

These data support a model in which SmgGDS-607 regulates the farnesylation of small GTPases, and this regulation is mediated by differential binding affinities. Although past studies have shown SmgGDS-607 regulates GTPases undergoing the geranylgeranylation pathway, we have shown that SmgGDS-607 regulation extends to the farnesylation pathway. Additionally, we have identified a novel function for SmgGDS-607 in facilitating product release of the farnesylated HRas (and possibly other small GTPases lacking a PBR) from protein farnesyltransferase. Further investigation into the synergies between the PBR and CAAAX identity for farnesylation and regulation should be explored to assess their potential as novel targets for anti-cancer therapeutics.

**Experimental procedures**

**Materials and methods**

Purified, His-tagged SmgGDS-607 was a gift from Dr. Benjamín C. Jennings (University of Michigan), Dr. Carol L. Williams (Medical College of Wisconsin) provided human DiRas1 cDNA that was subcloned into bacterial His6-tev and His6-tev-AviTagTM expression constructs. Dr. Arul M. Chinnaiyan (University of Michigan) provided human KRas4B and HRas cDNA that was subcloned into bacterial His6-tev and His6-tev-AviTagTM expression constructs. *Escherichia coli* BL21-A1 cells (Thermo Fisher Scientific) harboring pBirAcm (Avidity) and pRARE (from Rosetta Competent Cells; Millipore) plasmids were used to express biotinylated small GTPases.

**Preparation of *E. coli* human FTase and GGTase-I expression constructs**

Human FTase and GGTase-I expression plasmids were constructed in pETM-11 vectors with genes encoding the FTase subunits (pDG135) and the GGTase-I subunits (pDG140) in the order α–β. DNA fragments encoding the α subunit shared by both enzymes and the different β subunits for each enzyme were synthesized by Invitrogen. To construct plasmid pDG135, a pair of primers was designed to amplify the α subunit coding region (F1: 5’-ATTGTGCAGAGGATCAGATAGGCA-TCACCGTGTCGACTTATTGCTGGAC-TCGACGAGGATCAGGAAGATGAACG; and F6: 5’-CAATCTCGAGTCAAGCA-TCCGGTCGCCGTTCCG). Another pair of primers was used to amplify the FTase β subunit (F3: 5’-ATGCGCTGCGCGCCGACG; and F4: 5’-CTTGTCGACTTATTGCTGGACTGTT). The resulting PCR product for the α subunit was digested with Sall/Xhol, and the resulting PCR product for the β subunit was digested by Sall/NcoI. The two DNA fragments were then ligated into a Ncol/Xhol-digested pETM-11 vector.

To construct plasmid pDG140, a pair of primers was designed to amplify the GGTase-I β subunit coding region (F5, 5’-CAATAAGTGCAGAGGATCAGATAGGCA-TCACCGTGTCGACTTATTGCTGGAC-TCGACGAGGATCAGGAAGATGAACG; and F6, 5’-CTTGTCGACTTATTGCTGGACTGTT). The resulting PCR product for the β subunit was digested with Sall/EcoRI. The DNA fragment was then ligated into Sall/EcoRI-digested pDG135, which contains the shared α subunit between FTase and GGTase-I. DNA sequencing verified the protein sequence.

**FTase and GGTase-I expression and purification**

Recombinant human FTase was overexpressed and purified as previously described with a few modifications (41). BL21(DE3) cells were transformed with pDG135 plasmid for His6-tagged protein. The cells were grown in 2 liters of TB medium (24 g of tryptone, 48 g of yeast extract, 7.5 g of NaCl, 0.4% glycerol, 10 μM MgCl₂, 0.17 M KH₂PO₄, 0.72 M K₂HPO₄) supplemented with kanamycin (50 μg/ml) and 100 μM ZnCl₂ at 37 °C until reaching an A₆₀₀ of 1. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.8 mM to induce protein expression. The cells were grown for an additional 18 h postinduction at 25 °C until harvesting (10,000 × g for 30 min at 4 °C). The cell pellet was stored at −80 °C until purification.
Cell pellets were resuspended in chilled lysis buffer (20 mM Tris, pH 7.6, 200 mM NaCl, 5% glycerol, 5 mM imidazole, 2 mM tris(2-carboxyethyl)phosphine (TCEP)) supplemented with Pierce protease inhibitor mixture tablets (Thermo Fisher). The resuspended cells were lysed using a microfluidizer (Microfluidics). Nucleic acids were precipitated by the addition of 0.1% polyethyleneimine, and the lysate was cleared by centrifugation (35,000 × g for 25 min at 4 °C). The supernatant was then loaded on a 4-ml His-Pur™ Ni-NTA affinity column (Thermo Scientific). The column was washed with 5 column volumes of wash buffer (20 mM Tris, pH 7.6, 200 mM NaCl, 10 μM ZnCl₂, 5% glycerol, 10 mM imidazole, 1 mM TCEP), and protein was eluted with a stepwise imidazole gradient (25, 50, 75, 100, 150, 200, and 250 mM imidazole). The fractions were pooled and dialyzed overnight against buffer containing 20 mM Tris, pH 7.6, 200 mM NaCl, 10 μM ZnCl₂, 5% glycerol, and 1 mM TCEP. Protein was concentrated using Amicon Ultra concentrator (30-kDa MCMO, Millipore), aliquoted, and stored at −80 °C.

Human GGTase-I was expressed and purified similarly to FTase with a few modifications. BL21(DE3) cells were transformed with pDG140. Protein expression was induced at 22 °C until harvesting (10,800 g for 40 min at 4 °C), the supernatant was batch loaded onto a 500-ml His-Pur™ Ni-NTA affinity column and eluted with a stepwise imidazole gradient (25, 50, 75, 100, 150, 200, and 250 mM imidazole). After His-tag cleavage, the sample was centrifuged (2,700 × g for 15 min at 4 °C) to remove precipitated TEV protease. Cleaved GGTase-I was loaded onto a 4-ml His-Pur™ Ni-NTA affinity column with an imidazole stepwise gradient as described for FTase, active fractions were pooled, TEV protease was added at a 1:10 ratio (mg TEV:mg GGTase-I), and the sample was dialyzed (20-kDa MCMO) overnight. After His-tag cleavage, the sample was centrifuged (2,700 × g for 15 min at 4 °C) to remove precipitated TEV protease. Cleaved GGTase-I was loaded onto a 4-ml His-Pur™ Ni-NTA affinity column and eluted with a stepwise imidazole gradient (0, 10, 20, 40, 80, 160, and 300 mM imidazole). Active fractions were pooled, concentrated, aliquoted, and stored at −80 °C.

**Biotinylated small GTPase expression and purification**

The expression and purification of recombinant biotinylated small GTPases was completed as previously described with a few modifications (42). BL21-A1 cells harboring pBirAcm and pRARE plasmids were co-transformed with recombinant vector (pDG137, pDG138, pDG141, and pDG143) for His₆-tev-Avitag™ GTPase (KRas4B, KRas4B M188L, DiRas1, and HRas) and then grown at 37 °C in 50 ml of TB medium supplemented with kanamycin (25 μg/ml), chloramphenicol (10 μg/ml), and streptomycin (50 μg/ml). After reaching an A₆₅₀ of 0.6, the culture was cooled to 22 °C for 1 h. Protein expression was induced by adding 1.5 mM IPTG, followed by the addition of 0.1% biotin in Bicine buffer (pH 8.3) and 0.2% arabinose. The cells were grown for an additional 18 h postinduction at 22 °C until harvesting (2,700 × g for 30 min at 4 °C). The bacterial pellet was resuspended in lysis buffer (25 mM Tris, pH 7.6, 300 mM NaCl, 5% glycerol, 5 mM imidazole, 2 mM benzamidine, 1 mM PMSF, 1 μM pepstatin), and cells were lysed by sonication. After the lysate was cleared by centrifugation (10,800 × g for 40 min at 4 °C), the supernatant was batch loaded onto a 500-μl His-Pur™ Ni-NTA resin suspension for 2 h while shaking at 4 °C. The resin was transferred to a disposable Poly-Prep chromatography column (Bio-Rad) and washed with lysis buffer. Biotinylated protein bound to the resin was eluted with lysis buffer containing 300 mM imidazole. After protein elution, TEV protease was added at a 1:10 ratio (mg TEV:mg GTPase), and the sample was dialyzed (3.5-kDa MCMO) against 20 mM HEPES, pH 7.8, 200 mM NaCl, 5% glycerol, and 1 mM TCEP. After cleavage, the sample was centrifuged (2,700 × g for 15 min at 4 °C) to remove precipitated TEV protease. The supernatant was batch loaded to 500 μl of His-Pur™ Ni-NTA resin suspension for 1 h with shaking at 4 °C. Cleaved protein was batch eluted with 2 column volumes of lysis buffer in a stepwise imidazole gradient (0, 100, and 300 mM). Fractions were analyzed by SDS-PAGE. Protein fractions were pooled, aliquoted, and stored at −80 °C.

**Nonbiotinylated small GTPase expression and purification**

Small GTPases were expressed in BL21(DE3) E. coli cells cultured in 2 liters of LB medium (20 g of tryptone, 10 g of yeast extract, 20 g of NaCl, 10 mM MgCl₂, 0.5% glucose, 50 μg/ml kanamycin) by transforming with recombinant vectors encoding for KRas4B, KRas4B M188L, DiRas1, and HRas (pBJ176, pDG136, pBJ173, and pBJ162). The cells were grown at 34 °C until A₆₅₀ of 0.6–0.8. The culture was cooled to 18 °C, and protein expression was induced with addition of 0.1 mM IPTG. The cells were grown for an additional 18 h postinduction at 18 °C until harvesting (10,000 × g for 30 min at 4 °C). The cell pellet was stored at −80 °C until purification.

Following protein expression, recombinant small GTPases were purified similarly to FTase with a few modifications. After protein elution from a His-Pur™ Ni-NTA column with an imidazole stepwise gradient, the fractions were analyzed by SDS-PAGE, and fractions containing desired protein were pooled. TEV protease was added at a 1:10 ratio (mg TEV:mg small GTPase), and the sample was dialyzed (20-kDa MCMO) overnight against buffer containing 20 mM HEPES, pH 7.8, 200 mM NaCl, 5% glycerol, and 1 mM TCEP. After His-tag cleavage, the sample was centrifuged (2,700 × g for 15 min at 4 °C) to remove precipitated TEV protease. Cleaved protein was loaded onto a 4-ml His-Pur™ Ni-NTA affinity column and eluted with a stepwise imidazole gradient, as described for GGTase-I. Protein fractions were pooled, concentrated using Amicon Ultra concentrator (10-kDa MCMO, Millipore), aliquoted, and stored at −80 °C.

**Radiolabel prenylation assay**

Recombinant small GTPases were incubated in assay buffer (50 mM HEPPSO, pH 7.8, 5 mM TCEP, 5 mM MgCl₂, 0.1% Triton X-100) with GDP before addition of recombinant human FTase and varying concentrations of SmgGDS-607. Tritium-labeled farnesyl diphosphate ([3H]FPP, American Radiolabeled Chemicals Inc.) was diluted to 10% with unlabeled FPP in assay buffer. The reactions were initiated by the addition of radiolabeled FPP mixture and incubated at 30 °C before quenching the reactions by the addition of Laemmli sample buffer. Incubation times for the farnesylation reaction varied depending on the small GTPase to ensure the measurement was under initial velocity conditions (<10% reaction): KRas4B (5 min), KRas4B M188L (30 min), DiRas1 (6 min), and HRas (5 min). Unless indicated otherwise, the final concentrations were 2.5 μM small GTPase, 25 μM GDP, 25 μM FTase, and 4 μM [3H]FPP. After quenching, the samples were heated for 2 min at 70 °C before resolving by
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SDS-PAGE, followed by Coomassie Blue staining and destaining. The small GTPase bands were cut out, placed in scintillation vials, and dissolved by incubating in 500 μL of 34% H2O2, 0.2 mM CuSO4 at 35 °C overnight. The samples were counted on a Beckman LS 6500 liquid scintillation counter after addition of 4.5 ml of BioSafe II scintillation mixture (Research Products International). The pmol of prenylated product was determined using the specific activity (dpm/pmol) of tritium.

For steady-state kinetic studies, activity was measured by an increase in radioactivity upon farnesylation of the small GTPase. Assays were performed at 30 °C in 50 mM HEPES, pH 7.8, 5 mM TCEP, 5 mM MgCl2,2 0 mM GDP. FTase (50 nM) and HRas (0.5, 1, 2.5, 5, or 10 μM) were incubated in the absence or presence of 10 μM SmgGDS-607. The reactions were initiated by the addition of radiolabeled FPP at twice the concentration of HRas and incubated at 30 °C before quenching the reactions at various time points with Laemmli sample buffer. The initial rate of incorporation of radioactivity was determined by fitting a line to the time dependence of product formation, resulting in units of relative fluorescence units (RFU)/s. To obtain the initial rate of farnesylation in units of μM/s, Equation 3 was used, where \( V \) is the initial velocity of the reaction in units of μM/s, \( R \) is the initial velocity of the reaction in units of RFU/s, \( P \) is the max concentration of the substrate, and \( F_{\text{max}} \) is the max fluorescence intensity at the end point.

\[
V = \frac{RP}{F_{\text{max}}}
\]  
(Eq. 3)

The values for \( k_{\text{cat}} \) and \( K_m \) were determined by fitting the Michaelis–Menten equation to the concentration dependence of the initial velocity (\( V/E \)) using nonlinear regression in GraphPad Prism with the standard errors reported.

**Binding assay**

Binding affinities were measured by BLI using the Octet-Red96 instrument (Forte Bio). The assays were performed at 30 °C in 96-well plates. Streptavidin biosensors were loaded with biotinylated KRas4B, KRas4B M188L, DiRas1, or HRas in 50 mM HEPES, pH 7.8, 150 mM NaCl, 2 mM MgCl2, 20 μM GDP, 2 mM TCEP, and 0.25 mg/ml BSA. The loaded biosensors were washed in the same buffer before cycling through increasing concentrations of SmgGDS-607 or FTase. For FTase, the binding studies were performed in the presence of the FPT inhibitor II (Millipore), an inactive FPP analogue, at double the concentration of FTase. Controls included a sensor probe without biotinylated small GTPase that was incubated with either SmgGDS-607 or FTase and a sensor probe loaded with biotinylated small GTPase that was incubated only with buffer. Controls were subtracted from the binding data to correct for nonspecific binding. Kinetic parameters \( k_{\text{on}} \) and \( k_{\text{off}} \) were determined by fitting Equations 4 and 5, respectively, to the time dependence of complex association and dissociation. \( R \) refers to the BLI response (nm), \( IR \) is the initial BLI response (nm), \( Amp \) represents the amplitude of the BLI response change, \( k_{\text{on}} \) (M\(^{-1}\) s\(^{-1}\)) represents the rate constant of complex formation, \( k_{\text{off}} \) (s\(^{-1}\)) represents the rate constant of complex dissociation, and FR (nm) is the final BLI response.

\[
R = IR + Amp(1 - e^{-k_{\text{off}}})
\]  
(Eq. 4)

\[
R = Amp(e^{-k_{\text{off}}}) + FR
\]  
(Eq. 5)

The dissociation constant, \( K_D \), was determined by fitting the responses at equilibrium (\( R_{\text{eq}} \)) for each SmgGDS-607 or prenyltransferase concentration to a binding isotherm (Equation 6), where \( K_D \) is the dissociation constant, and \( X \) is the concentration of either SmgGDS-607 or prenyltransferase. The data were fit using nonlinear regression in GraphPad Prism with the standard errors reported.

\[
R_{\text{eq}} = \frac{R_{\text{max}}[X]}{K_D + [X]}
\]  
(Eq. 6)

**Peptide prenylation assay**

For steady-state kinetic studies performed with peptide, FTase activity was measured by an increase in fluorescence intensity (λ\(_{\text{ex}}\) = 340 nm, λ\(_{\text{em}}\) = 520 nm) upon farnesylation of dansylated peptide using a previously published assay (27). Experiments were carried out using dansyl-TKCVI peptide, which mimics the CAAX box of KRas4B. Assays were performed at 30 °C in 50 mM HEPES, pH 7.8, 5 mM TCEP, 5 mM MgCl2, and 20 μM FPP. FTase (50 nM) and dansyl-TKCVIM (0.2, 0.4, 0.8, 1.6, 2.4, and 4.8 μM) were incubated in the absence or presence of 5 μM SmgGDS-607. The reactions were measured every 30 s for 1.5 h. The initial rate of farnesylation was determined by fitting a line to the time dependence of product formation, resulting in units of relative fluorescence units (RFU)/s. To obtain the initial rate of farnesylation in units of μM/s, Equation 3 was used, where \( V \) is the initial velocity of the reaction in units of μM/s, \( R \) is the initial velocity of the reaction in units of RFU/s, \( P \) is the max concentration of the substrate, and \( F_{\text{max}} \) is the max fluorescence intensity at the end point.

\[
V = \frac{RP}{F_{\text{max}}}
\]  
(Eq. 3)

The values for \( k_{\text{cat}} \) and \( K_m \) were determined by fitting the Michaelis–Menten equation to the concentration dependence of the initial velocity (\( V/E \)) using nonlinear regression in GraphPad Prism with the standard errors reported.

**Author contributions**—D. G.-T. and C. A. F. conceptualization; D. G.-T. and C. A. F. formal analysis; D. G.-T. investigation; D. G.-T. methodology; D. G.-T. writing—original draft; D. G.-T. and C. A. F. writing—review and editing; C. A. F. funding acquisition; C. A. F. project administration.
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