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Secramine inhibits Cdc42-dependent functions in cells and Cdc42 activation *in vitro*

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Inspired by the usefulness of small molecules to study membrane traffic, we used high-throughput synthesis and phenotypic screening to discover secramine, a molecule that inhibits membrane traffic out of the Golgi apparatus by an unknown mechanism. We report here that secramine inhibits activation of the Rho GTPase Cdc42, a protein involved in membrane traffic, by a mechanism dependent upon the guanine dissociation inhibitor RhoGDI. RhoGDI binds Cdc42 and antagonizes its membrane association, nucleotide exchange and effector binding. *In vitro*, secramine inhibits Cdc42 binding to membranes, GTP and effectors in a RhoGDI-dependent manner. In cells, secramine mimics the effects of dominant-negative Cdc42 expression on protein export from the Golgi and on Golgi polarization in migrating cells. RhoGDI-dependent Cdc42 inhibition by secramine illustrates a new way to inhibit Rho GTPases with small molecules and provides a new means to study Cdc42, RhoGDI and the cellular processes they mediate.

Intracellular membrane traffic, organelle movement and cytoskeleton dynamics are rapid, highly regulated cellular processes. For example, proteins destined for secretion are shuttled from the endoplasmic reticulum (ER), their site of synthesis, to the plasma membrane through the Golgi apparatus, often in less than 3 h. The speed and complexity of these events make them challenging to study by genetic and biochemical means. Small molecules can act very rapidly and can be extraordinarily useful tools, particularly if they are cell permeable, are specific and act reversibly. For example brefeldin A, exo1 and exo2, which each induce a marked redistribution of Golgi proteins into the ER and disassembly of the Golgi, have helped to reveal transport pathways from the Golgi and the *trans*-Golgi network (TGN) to the ER[1–3].

The Rho family of small GTPases (Rho GTPases), of which the best-characterized members are RhoA, Rac1 and Cdc42, participate in membrane traffic and Golgi dynamics[4–6] as well as in other essential cellular processes, such as cell motility, cell adhesion and cytokinesis, probably by regulating the actin cytoskeleton[7,8]. Other small GTPases, such as those of the Arf and Rab families, also participate in membrane transport events. Arf GTPases recruit vesicle budding machinery, and Rab GTPases ensure proper vesicle targeting. Like Rho GTPases, these enzymes cycle between the cytosol and membranes and bind and hydrolyze GTP.

In mammalian cells, the Golgi apparatus is a juxtanuclear ribbon of interconnected, membrane-enclosed stacks that communicate through vesicle- and tubule-mediated transport. Actin and microtubule-based filament networks associate with the Golgi stacks and, together with the Golgi matrix proteins, regulate both positioning and transport events. Although useful for investigating transport between the ER and Golgi apparatus, the small molecules brefeldin A, exo1 and exo2 each disrupt the characteristic localization and morphology of the Golgi. Thus, they are generally not useful for studying traffic emanating from the Golgi. To discover new small molecules that perturb protein traffic out of the Golgi, we used diversity-oriented synthesis and image-based phenotypic screening. Recognizing the impact of natural products like brefeldin A in the study of biological processes, we chose to synthesize a library of complex, natural product–like small molecules resembling galanthamine. From the library, we found a fast-acting compound that inhibits transport from the Golgi and called it secramine (referred to here as secramine A, **1**)[9]. Here we describe experiments that identify the Rho GTPase Cdc42 and its association with Rho guanine nucleotide dissociation inhibitor 1 (RhoGDI1) as a principal target for secramine A and related molecules. We observed that the effects of secramine A on transport from the Golgi are similar to those induced by cytochalasin B and those induced by expression of Cdc42 mutants. Therefore, we designed *in vitro* assays involving extracts and pure constituents and found that secramine A prevents Cdc42 activation (GTP loading) by reducing the binding of prenylated Cdc42 to membranes. These processes are strictly dependent on the presence

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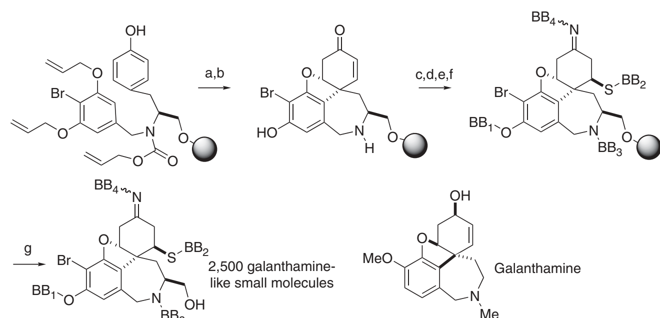

Scheme 1 Outline of synthesis leading to secramine A and other galanthamine-like library members[9]. BB = building block (sites of variability among compounds). The structure of galanthamine is also shown. Reagents: a, $PhI(OAc)_2$, $(CF_3)_2CHOH-CH_2Cl_2$, 23 °C; b, $Pd(PPh_3)_4$, morpholine-THF, 23 °C; c, BB_1OH, PPh_3, DIAD, THF, 0 °C (2×); d, BB_2SH, 2,6-lutidine, *n*-BuLi, THF, 0 °C to 40 °C; e, BB_3CHO, AcOH, MeOH-THF then $NaBH_3CN$, 23 °C or BB_3COCl, 2,6-lutidine, CH_2Cl_2, 23 °C or BB_3NCO, CH_2Cl_2, 23 °C; f, BB_4NH_2, AcOH, $MeOH-CH_2Cl_2$, 23 °C; g, HF.pyr, THF, 23 °C, then TMSOMe.

of RhoGDI1, which shuttles Cdc42 between the cytosol and membranes. We propose a model for the molecular mechanism of how secramine A inhibits Cdc42 activation. We further show that secramine A can be used to study other processes that require Cdc42: for example, nuclear orientation during cell migration. These studies independently corroborate a role for RhoGDI1 and Rho GTPases in regulating membrane traffic at the Golgi and demonstrate a new fast-acting reagent for investigation of these proteins.

RESULTS

Secramine inhibits anterograde transport from the Golgi

We synthesized a library of ~2,500 complex small molecules by mimicking the biosynthesis of the natural product galanthamine on polystyrene beads (**Scheme 1**)[9]. Library members were eluted from the beads and, using automated fluorescence microscopy, were directly screened to identify inhibitors of the ER-to-Golgi-to–plasma membrane transport of a viral glycoprotein fused to green fluorescent protein ($VSVG^{ts}$-EGFP; **Fig. 1a**)[2,9,10]. This chimera accumulated in the ER at the nonpermissive temperature of 40 °C. Upon cooling to 32 °C, it synchronously passed in 2.5 h from the ER to the plasma membrane via the Golgi apparatus (**Fig. 1a**). For the primary screen, compounds were pin transferred into 384-well plates containing monkey kidney epithelial (BS-C-1) cells expressing $VSVG^{ts}$-EGFP at 40 °C. The plates were then incubated at 32 °C for 2.5 h, fixed, imaged and scored by visual inspection. Of the 114 compounds found to inhibit Golgi-to–plasma membrane transport, over 50% had a secondary amine at building block position 3 (BB_3), whereas fewer than 10% had an amide or urea at BB_3 (**Supplementary Fig. 1** online). Five compounds, each with a secondary amine (BB_3) and oxime (BB_4), retained activity when rescreened at a tenfold dilution (**Fig. 1a**). Secramine A appeared to be the most potent of these compounds. In agreement with our screening results, replacement of the *O*-benzyloxime of secramine A with an *O*-methyloxime (labeled in red), resulting in

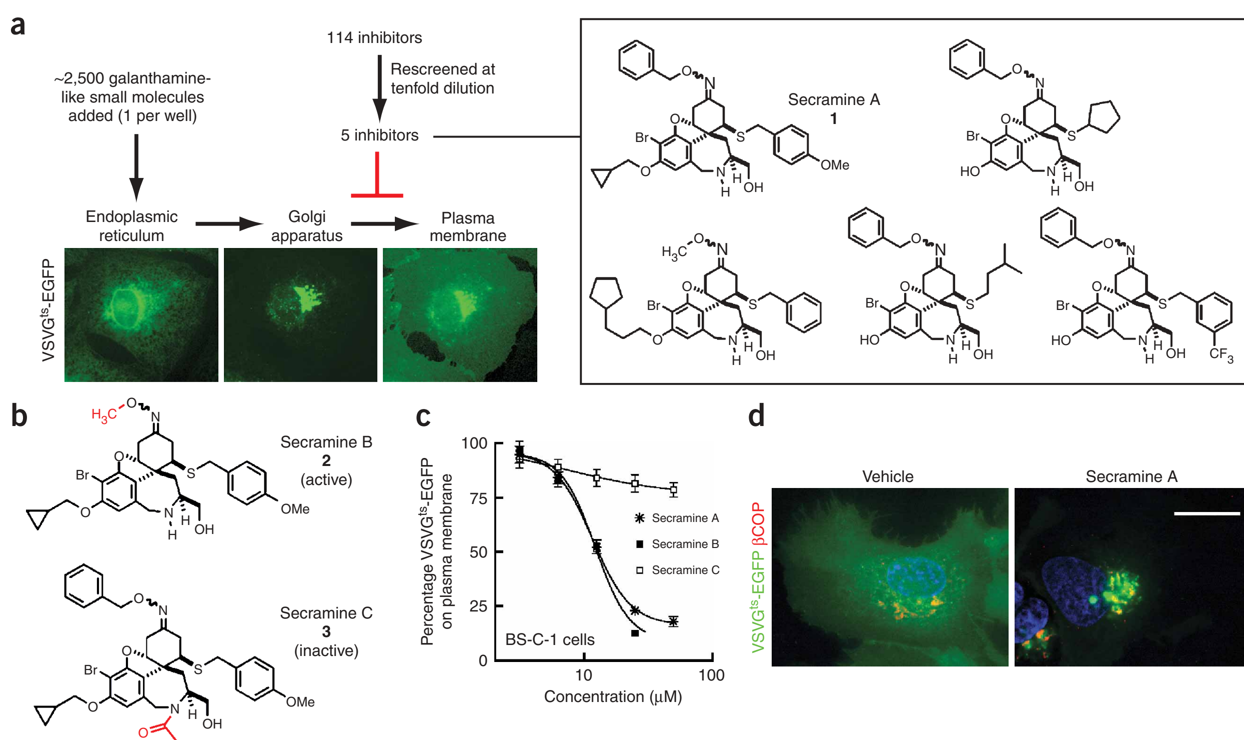

Figure 1 Screening of a library of galanthamine-like small molecules leading to the identification of secramine A as an inhibitor of Golgi-to–plasma membrane transport. (**a**) Outline and results of the membrane traffic screen. Initially, 114 compounds inhibited the Golgi-to–plasma membrane transport of $VSVG^{ts}$-EGFP. The library was rescreened, and five compounds retained activity (shown in box at right). (**b**) Structures of secramines B and C. Structural differences are highlighted in red. (**c**) Effect of secramines A, B and C on the delivery of $VSVG^{ts}$-EGFP from the ER to the plasma membrane. Secramines A and B had an IC_{50} of ~12 μM. Vertical bars represent s.e.m. ($n = 4$). (**d**) In monkey kidney epithelial (BS-C-1) cells, $VSVG^{ts}$-EGFP (green) accumulated at the plasma membrane 3 h after transport from the ER (vehicle, 1% DMSO) or remained in the perinuclear region (12.5 μM secramine A), partially colocalizing with the Golgi marker β-COP (red). The nucleus was labeled with Hoechst dye (blue). Scale bar, 20 μm.

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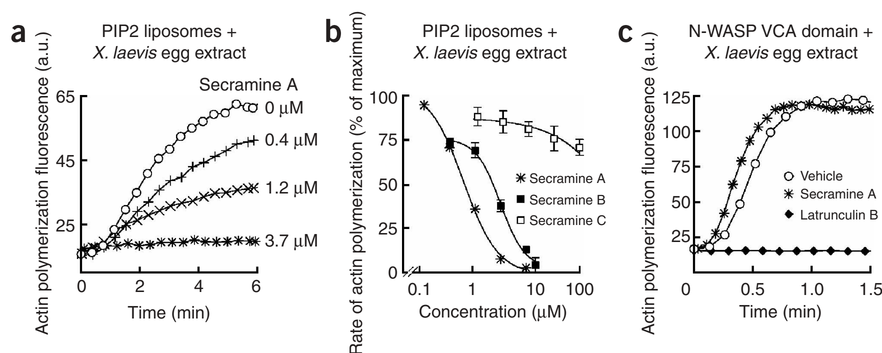

Figure 2 Secramine A inhibits PIP2 liposome–stimulated actin polymerization in *X. laevis* egg extract and acts upstream of Arp2/3 complex activation. (**a**) Inhibitory effect of secramine A on the polymerization kinetics of pyrene-actin after the addition of 10 μM PIP2 liposomes into the *X. laevis* egg extract. (**b**) Effect of secramines A, B and C on the maximum rate of PIP2 liposome–stimulated pyrene-actin polymerization. For secramines A and B, IC_{50} was ∼1 μM and ∼3 μM, respectively. Vertical bars represent s.e.m. ($n = 3$). (**c**) Polymerization kinetics of pyrene-actin after the addition of 100 nM VCA domain of N-WASP to *X. laevis* egg extract treated with vehicle (1% DMSO), 20 μM secramine A or 10 μM latrunculin B.

secramine B (**2**), did not alter potency, whereas acetylation of the secondary amine of secramine A (labeled in red) to generate an acetamide, secramine C (**3**), strongly diminished activity (**Fig. 1b**,**c**). Secramines A, B and C were resynthesized in solution after the solid-phase library synthesis route and were used in all subsequent studies (**Supplementary Methods** online).

Secramine A caused retention of VSVG^{ts}-EGFP in the Golgi, as indicated by its partial colocalization with β-COP, a subunit of the COPI coatomer (**Fig. 1d**). In contrast, secramine A did not affect ER-to-Golgi transport or the functional integrity of the Golgi, as determined by fluorescence imaging and by monitoring the kinetics of carbohydrate processing of VSVG^{ts}-EGFP as it entered the Golgi apparatus (**Supplementary Videos 1** and **2** and **Supplementary Fig. 2** online). The retention of VSVG^{ts}-EGFP in the Golgi is reversible upon removal of secramine A (**Supplementary Video 3** and **Supplementary Fig. 2** online). Secramine A also did not affect perinuclear localization of the TGN, Golgi-to-ER transport or endocytosis of transferrin[9] (**Supplementary Fig. 2**).

Secramine inhibits actin polymerization

We considered potential mechanisms for inhibition of VSVG^{ts}-EGFP transport from the Golgi by secramine A, including the possibility that secramine A interferes with actin dynamics, because cytochalasin B, a small molecule that prevents actin polymerization, also delays VSVG^{ts}-GFP transport from the Golgi[11]. We tested the impact of secramine A on actin dynamics by monitoring actin polymerization in *Xenopus laevis* cytoplasmic egg extract (referred to as *X. laevis* egg extract) stimulated with liposomes containing phosphatidylinositol 4,5-bisphosphate (referred to as PIP2 liposomes)[12]. PIP2 liposomes stimulate actin polymerization through an evolutionarily conserved pathway involving the activation of (i) Cdc42, (ii) the Cdc42 effectors Toca-1 and N-WASP and (iii) the Arp2/3 complex[12–14]. The Arp2/3 complex directly binds to actin and nucleates actin filament assembly. The PIP2 liposomes were composed of a 48:48:4 molar mixture of phosphatidylcholine (PC) to phosphatidylinositol (PI) to PIP2. We monitored actin polymerization by the increase in fluorescence of actin-pyrene as it incorporates into actin filaments[14,15]. Secramines A and B inhibited this pathway, but secramine C did not (**Fig. 2a**,**b**), matching their relative activity in cells.

Target of secramine

The consistent relative activities of secramine analogs in actin polymerization and membrane traffic suggest that secramine A inhibits the same target in both processes. We therefore dissected the more experimentally tractable actin polymerization pathway to search for the molecular target of secramine A. To test whether secramine A directly interferes with the Arp2/3 complex or actin, we initiated actin polymerization with the N-WASP VCA domain, which bypasses the requirement for other factors upstream of N-WASP[14]. Unlike latrunculin B, a small molecule that binds to actin, secramine A did not prevent actin assembly induced by the VCA domain (**Fig. 2c**). Thus, secramine A does not perturb actin or the Arp2/3 complex directly, but rather interferes with a component in the signaling pathway between PIP2 and N-WASP.

To probe the actin pathway upstream of the Arp2/3 complex, we tested whether secramine A inhibits PIP2 liposome–stimulated Cdc42 activation. Cdc42 is considered active when it is bound to GTP and free to bind its effectors, such as N-WASP and PAK1 (**Fig. 3a**)[16]. A recombinant polypeptide derived from PAK1 (GST-PBD) can be immobilized on beads and used to specifically precipitate GTP-bound Cdc42 and Rac[17]. Using this approach, we found that secramines A and B inhibited the association of Cdc42(GTPγS) with GST-PBD, whereas secramine C did not (**Fig. 3b**). We concluded that secramine A inhibits either the interaction between Cdc42(GTPγS) and GST-PBD (**Fig. 3a**, step 5), the loading of GTPγS onto Cdc42 by a guanine nucleotide exchange factor (GEF; **Fig. 3a**, step 3), or the association of Cdc42 with PIP2 liposomes (**Fig. 3a**, steps 1 or 2). We tested the binding between recombinant nonprenylated Cdc42(GTPγS) and GST-PBD and observed no interference by secramine (**Fig. 3c**, lane 1) even when used at 50 μM, a concentration well above its maximal effect on endogenous Cdc42 (**Fig. 3b**, lane 3). Therefore, secramine A does not directly prevent GST-PBD binding to Cdc42. To initially test for an inhibition of GEF activity, we performed an 'order of addition' experiment, also at 50 μM secramine A (**Fig. 3d**). We mixed *X. laevis* egg extract with GTPγS and PIP2 liposomes to generate Cdc42(GTPγS) through the activity of an endogenous GEF. Cdc42(GTPγS) was precipitated by its interaction with GST-PBD and detected by western blotting with a Cdc42-specific antibody (**Fig. 3d**, lane 2). Addition of secramine A after Cdc42(GTPγS) formation inhibited Cdc42(GTPγS) recovery (**Fig. 3d**, lane 4), but addition of secramine A after adding GST-PBD did not (**Fig. 3d**, lane 5). These results indicated that secramine A indirectly blocks endogenous Cdc42(GTPγS) from binding GST-PBD, possibly by inhibiting the membrane association of Cdc42.

Secramine inhibits membrane-dependent Cdc42 activation

To support the conclusion that secramine A inhibits Cdc42 membrane association in cellular extracts, we reconstituted the membrane-dependent nucleotide exchange activity of prenylated Cdc42 *in vitro* using pure components (**Fig. 4a–e**). PIP2 liposomes and the isolated Dbl homology and plekstrin homology (DH-PH) domain of the GEF Dbl's big sister (Dbs), when mixed with the Cdc42(GDP)-RhoGDI1 complex and excess RhoGDI1, enabled the rapid exchange of GDP for

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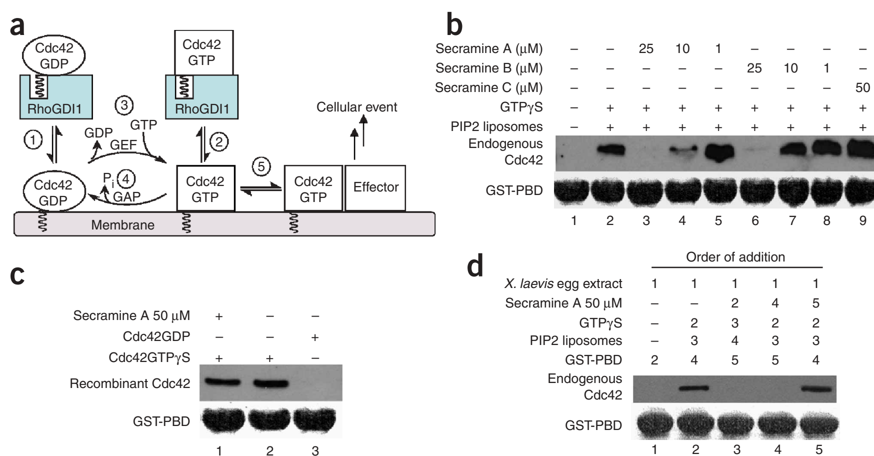

Figure 3 Secramine A inhibits Cdc42 activation in *X. laevis* egg extract. (**a**) Model for the regulation of Cdc42 (ref. 16). RhoGDI1 translocates Cdc42 between the cytosol and target membranes (steps 1,2). Guanine nucleotide exchange factor (GEF) catalyzes GDP release (step 3). GTPase activating protein (GAP) catalyzes GTP hydrolysis (step 4). Cdc42(GTP) interacts with effectors such as N-WASP and PAK1 to engage signaling pathways (step 5). (**b**) Effect of secramines A, B and C on the activation of Cdc42 by GTPγS and PIP2 liposomes, as measured by its precipitation with GST-PBD. Secramines A, B and C were mixed with *X. laevis* egg extract before the addition of GTPγS, PIP2 liposomes and GST-PBD. Cdc42 was detected by western blot, and, as a loading control, GST-PBD was detected by staining with Ponceau S. (**c**) Secramine A does not inhibit the binding of recombinant nonprenylated Cdc42(GTPγS) to GST-PBD in *X. laevis* egg extract. (**d**) Secramine A inhibits Cdc42 activation if added before GST-PBD. Numbers indicate the order in which the various components were added.

GTPγS on Cdc42. It has been observed that PIP2 liposomes support the dissociation of geranylgeranylated Cdc42(GDP) from RhoGDI1 and bind to geranylgeranylated Cdc42(GTPγS)[18]. In agreement with these observations, we found that liposomes composed entirely of PC or a 2:1 ratio of PC to PI did not significantly stimulate nucleotide exchange (data not shown). This reaction was inhibited by secramines A and B, but not by secramine C. Secramines A and B did not interfere directly with the GEF, nor did they compete with nucleotide binding, as nucleotide exchange of nonprenylated Cdc42, which does not require membrane association, was insensitive to secramines A and B (**Fig. 4b**). To achieve a similar rate of nucleotide exchange to that observed with prenylated Cdc42, the reaction required a tenfold higher concentration of GEF, probably because binding of Cdc42 and GEF to the liposome surface markedly increases their effective concentration relative to the solution phase interaction of nonprenylated Cdc42 and GEF. Furthermore, we determined that secramines A and B inhibited GEF-independent nucleotide exchange of prenylated Cdc42 by replacing the GEF with EDTA, a chelator of Mg^{2+} (**Fig. 4c,d**). Mg^{2+} stabilizes nucleotide binding to Cdc42, and its absence increases the intrinsic nucleotide exchange rate for Cdc42 (ref. 19). Secramines A and B did not inhibit the EDTA-stimulated nucleotide exchange of nonprenylated Cdc42 (**Fig. 4e**), again indicating that secramine A does not compete with nucleotide binding and inhibits nucleotide exchange only on prenylated Cdc42. Nucleotide exchange of prenylated Cdc42 requires membrane translocation and dissociation from RhoGDI1 (ref. 16). Indeed, secramines A and B decreased the membrane association of prenylated Cdc42(GDP) that was presented to PIP2 liposomes as a Cdc42(GDP)-RhoGDI1 complex (**Fig. 4f**).

Secramine does not act directly on PIP2

We considered the possibility that secramine A might inhibit Cdc42 activation and membrane association simply by acting as a detergent to solubilize PIP2 liposomes or by binding to the inositol head group of PIP2, making PIP2 unavailable for protein-lipid recognition. We ruled out detergent activity, as secramine A had no effect on the appearance of PIP2 liposomes imaged by negative-staining electron microscopy, whereas the detergent Triton X-100 completely dissolved them (**Fig. 5a**). To determine if secramine directly prevents PIP2 from binding to protein partners, we tested if secramine affected PIP2-dependent stimulation of GTP hydrolysis by the large GTPase dynamin. Neomycin, an antibiotic that interferes with PIP2-dependent processes by binding to the inositol head group of PIP2 (ref. 20), blocked the PIP2-dependent stimulation of GTP hydrolysis by dynamin 2 (ref. 21), whereas secramines A and B did not (**Fig. 5b**). Moreover, secramine A does not inhibit the endocytosis of transferrin, a PIP2-dependent process[22]. We conclude that secramine A does not act directly on PIP2.

Secramine inhibits Cdc42 binding to Golgi membranes

Activated Cdc42 associates with Golgi membranes[23,24]. This binding occurs downstream of the related GTPase Arf1, which recruits COPI coatomers to Golgi membranes. The γ subunit of COPI, in turn, recruits activated Cdc42 (ref. 25). *In vitro*, mixing of purified rat liver Golgi membranes with bovine brain extract and GTPγS leads to membrane association of Cdc42 and COPI (monitored by the recruitment of its subunit β-COP)[24,26]. Consistent with our observations on binding of Cdc42 to liposomes (**Fig. 4f**), secramines A and B inhibited Cdc42 but not β-COP binding to Golgi membranes. This result also provides evidence that secramine is specific for members of the Rho family rather than for members of the Arf family of GTPases (**Fig. 6a**).

Secramine inhibits traffic of a basolateral protein

Inhibition of Cdc42 function in cells by overexpression of a dominant-negative GEF-sequestering mutant form of Cdc42 disrupts basolateral membrane-directed protein transport from the Golgi in canine kidney epithelial (MDCK) cells[5,27]. To study the effects of secramine A on polarized secretion, we monitored the transport of C-terminally GFP-tagged neuronal cell adhesion molecule (NCAM-GFP) or neurotrophin receptor (p75-GFP), which follow basolateral and apical routes to the cell surface, respectively, in MDCK cells[27]. Overexpression of dominant-negative Cdc42 (N17Cdc42) delays NCAM-GFP transport but stimulates p75-GFP transport from the Golgi[27]. In our experiments, treatment with secramine A had similar effects: secramine A delayed NCAM-GFP transport and, albeit slightly, stimulated p75-GFP transport (**Fig. 6b**). This data also shows that the inhibitory effect of secramine A is fast, as it was observed after a 20-min incubation of the cells with secramine A.

Secramine disrupts Golgi polarization in migrating cells

In many cells undergoing polarized migration, the Golgi comigrates with the microtubule organizing center (MTOC) as it reorients between the nucleus and the leading edge of the cell; the MTOC

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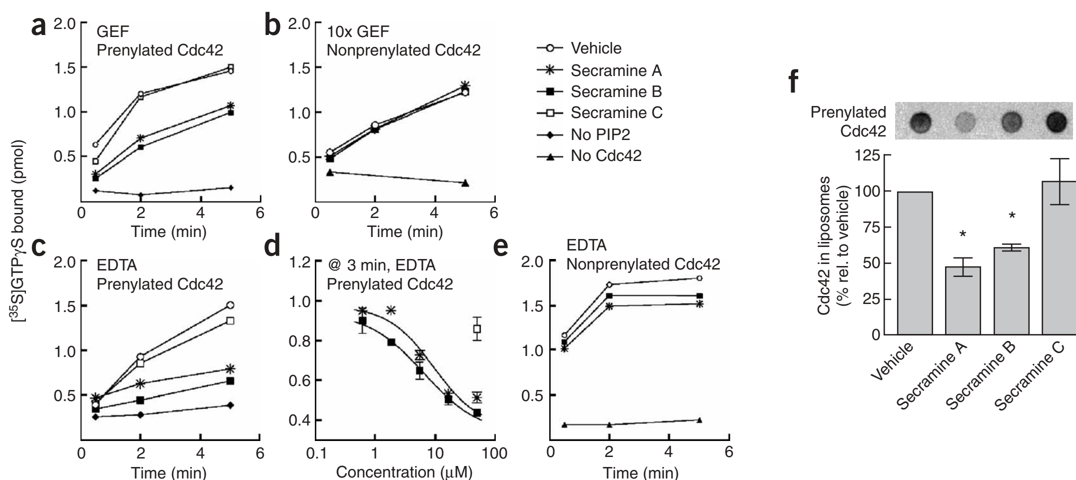

Figure 4 Secramine A inhibits Cdc42 activation and PIP2 liposome association *in vitro*. (**a**) Time course of [^{35}S]GTPγS binding to a mixture containing 0.5 μM Cdc42(GDP)-RhoGDI1 complex, 1 μM RhoGDI1, 100 μM PIP2 liposomes, 30 nM DH-PH domain of the GEF Dbs (GEF), and vehicle or 25 μM secramine. (**b**) As in **a**, but with 300 nM GEF, 1.5 μM RhoGDI1, and 0.5 μM nonprenylated Cdc42. (**c**) As in **a**, but with 4 mM EDTA instead of GEF. (**d**) As in **c**, with each reaction analyzed at 3 min. Secramines A and B have an IC_{50} of ~9 μM and ~7 μM, respectively. Vertical bars represent s.e.m. ($n = 3$). (**e**) As in **b**, except that 4 mM EDTA was used instead of the GEF. (**f**) Inhibitory effect of secramines A and B on the association of prenylated Cdc42 with PIP2 liposomes. We mixed 0.5 μM Cdc42-RhoGDI1 complex with 1 μM RhoGDI1, 1 mM PIP2 liposomes and either vehicle (2% DMSO) or 100 μM secramine. After 10 min, the PIP2 liposomes were sedimented. A representative dot blot of the pellet fraction is shown. The amount of Cdc42 was quantified and normalized to vehicle. Vertical bars represent s.e.m. ($n = 4$). *, $P < 0.05$ (compared with vehicle).

remains stationary while the nucleus moves away from the leading edge in a process regulated by activated Cdc42 (refs. 28,29). To establish that secramine inhibits other known Cdc42-dependent processes in living cells, we studied the effects of secramine A on this process. We scratched a monolayer of U373MG astrocytes and observed the relative position of the Golgi (probed with the Golgi matrix protein giantin) as it reoriented in cells located along the edge of the scratch. After 6 h, about 45% of the cells incubated with secramine A reoriented their Golgi in the forward direction toward the scratch, whereas 60% of the cells did so in the absence of secramine (**Fig. 6c,d**). This represented a significant inhibition ($P < 0.1$) of ~45%, as a random orientation occurs whenever 33% of cells locate their Golgi in the forward position. Thus, these results provide further support for the interference of secramine in Cdc42-dependent cellular processes.

DISCUSSION

Secramine A was originally identified as an inhibitor of VSVG transport from the Golgi apparatus to the plasma membrane (**Fig. 1**)[9]. We reasoned that secramine might perturb actin regulation, as treatment with cytochalasin B, an inhibitor of actin polymerization, also retards traffic from the Golgi[11] and has only minor effects on overall Golgi organization. Consistent with this hypothesis, we observed that secramine inhibits *in vitro* assembly of actin that is stimulated by PIP2 and mediated by the Cdc42/Toca-1/N-WASP/Arp2/3 signaling pathway (**Fig. 2**)[13]. We traced this inhibitory effect to interference in membrane recruitment of prenylated Cdc42 that ultimately prevents its activation (**Figs. 3** and **4**). The inhibitory effects of secramine were strictly dependent on the presence of RhoGDI1, a protein that promotes the solubility of prenylated Cdc42 and is believed to act as a shuttle for the transport of GDP-Cdc42 or GTP-Cdc42 between the cytosol and target membranes[16]. Whether secramine A prevents translocation of prenylated Cdc42 from RhoGDI1 to membranes or contributes to extraction of membrane-bound prenylated Cdc42 by RhoGDI1 remains to be determined.

We envision that secramine stabilizes the association of Cdc42 with RhoGDI1, thereby decreasing availability of Cdc42 for activation and downstream signaling. The crystal structure of the Cdc42-RhoGDI1 complex shows an extended interface with several potential sites for a small molecule to bind and stabilize the interaction[30]. This type of mechanism is similar to the mode of action of brefeldin A, a reagent that stabilizes a transient complex formed between the small GTPase Arf1 in its GDP-bound form and some of its

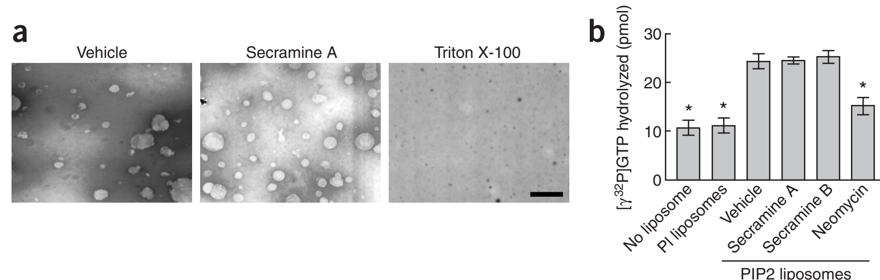

Figure 5 Secramine does not disrupt PIP2 liposomes. (**a**) PIP2 liposomes (100 μM) were treated with either vehicle (1% DMSO), 50 μM secramine A, or 1% Triton X-100 and were imaged by electron microscopy. Scale bar, 1 μm. (**b**) Effect of secramines A and B on PIP2 liposome stimulation of GTP hydrolysis by the large GTPase dynamin 2 (ref. 21). A truncated mutant of dynamin 2 lacking the C-terminal proline- and arginine-rich domain was mixed with either vehicle (1% DMSO), 25 μM secramines A or B, or 250 μM of neomycin, as indicated, and GTP hydrolysis was monitored by the presence of ^{32}P-labeled phosphate. Vertical bars represent s.e.m. ($n = 3$); *, $P < 0.05$ (compared with vehicle treatment of PIP2 liposomes).

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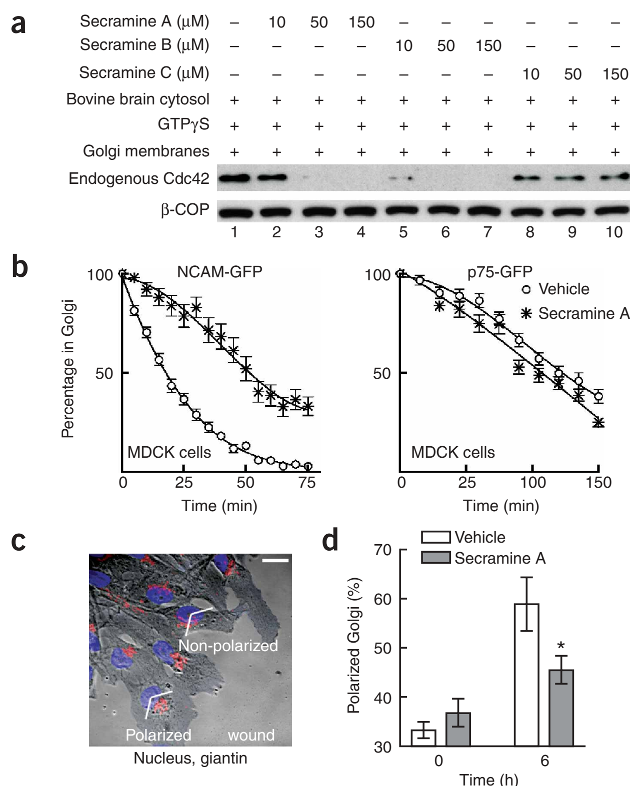

Figure 6 Effect of secramine A on Cdc42-dependent events. (**a**) Secramines A and B inhibit the *in vitro* binding of Cdc42 to Golgi membranes. Bovine brain cytosol was incubated with vehicle (1% DMSO) or secramine, followed by addition of 20 μM GTPγS and 0.2 mg ml^{-1} rat liver Golgi membranes[24]. The membranes were processed for western blot analysis[24]. (**b**) Secramine A (10 μM) delays the transport of NCAM-GFP but not p75-GFP from the Golgi of nonpolarized MDCK cells. GFP fluorescence in the perinuclear area was normalized to total cell fluorescence and expressed as a percentage of Golgi fluorescence at $t = 0$ (ref. 27). Vertical bars represent s.d. ($n = 2$; 20 cells per condition). (**c**,**d**) Effect of secramine A on polarization of the Golgi of astrocytoma cells. A confluent monolayer of U373MG human astrocytoma cells was incubated with vehicle or 15 μM secramine A for 1 h followed by scratching. At the indicated times, the cells were fixed and stained for the nucleus with Hoechst dye (blue) and for the Golgi with an antibody against giantin (red). (**c**) Representative view of vehicle-treated U373MG cells imaged by epifluorescence and phase contrast microscopy 8 h after wounding. Cells in which Golgi localized to the forward 120° sector towards the scratch were scored as polarized[28]. Bar, 20 μm. (**d**) Summary of analysis performed immediately after the scratch (0 h) and 6 h later. Vertical bars represent s.e.m. ($n = 3$, >100 cells per condition); *, $P < 0.1$ (compared with vehicle).

exchange factors, preventing GDP/GTP exchange[31,32]. Thus, Arf1-GTP is consumed and not replaced so processes that depend on Arf1-GTP, such as membrane recruitment or activation of various proteins involved in regulation of vesicular traffic, are blocked.

We can rule out that secramine A inhibits members of the related family of Rab GTPases, which, like Rho GTPases, are shuttled between the cytosol and membranes by a GDI (RabGDI). Not only are the RabGDI and RhoGDI structures dissimilar[33], but Rab GTPases are required for nearly all membrane traffic events, including endocytosis and ER-to-Golgi transport, neither of which is inhibited by secramine A. Does secramine A interfere with the activity of other Rho-family GTPases, such as RhoA and Rac1, that also bind to RhoGDI1 (ref. 16)? Secramine A (25 μM) had no discernable effect on the appearance of stress fibers in BS-C-1 cells after 3 h of treatment (data not shown), suggesting that in cells, secramine did not change the overall level of Rho activation[34]. Using GST-PBD to precipitate GTP-bound Rac from *X. laevis* egg extract, we found that secramine A also prevented PIP2 liposome–stimulated Rac1 activation (**Supplementary Fig. 3** online). Nevertheless, activation of Rac in this system has not been previously reported and may occur downstream of Cdc42 signaling, as observed in other systems[35]. Future studies using a reconstituted system with pure proteins should provide a definitive answer to this question.

The major pool of membrane-bound Cdc42 in mammalian cells is located in Golgi membranes[23]; it is retained in part through an association with the γ-subunit of COPI[25]. Thus, it is not surprising that interference with Cdc42 activity impinges on important aspects of Golgi function. Like secramine A, overexpression of a GEF-sequestering mutant form of Cdc42 (N17Cdc42) interferes with the transport of a basolateral protein from the Golgi[27] but does not interfere with retrograde transport from the Golgi to the ER[36]. As expected for an inhibitor of Cdc42 function, secramine A does not interfere with retrograde Golgi-to-ER transport of the KDEL receptor (**Supplementary Fig. 2**). Only activation of Cdc42 pathways, by overexpression of a Cdc42 mutant that fails to hydrolyze GTP (V12Cdc42), was observed to inhibit KDEL receptor traffic out of the Golgi[36]. Conflicting studies, based on use of dominant-negative N17Cdc42, report that Cdc42 does[25] and does not[36] regulate ER-to-Golgi transport of VSVG. Our results with secramine A support the conclusion that Cdc42 is not required for ER-to-Golgi transport of VSVG (**Supplementary Fig. 2** and **Supplementary Videos 1** and **2**).

We have shown that secramine inhibits established Cdc42-dependent processes in diverse species, and secramine analogs have consistent relative activities in cells, in extracts and *in vitro*. And unlike expression of dominant-negative Cdc42, treatment with secramine is acute, reversible and tuneable. In contrast to the small molecule geranylgeranyltransferase inhibitors that prevent prenylation of Cdc42 and other proteins[37], secramine A is fast acting and useful in cell-free systems, as it does not depend on the cellular degradation of the existing pool of prenylated Cdc42. Moreover, secramine A does not directly inhibit GEF activity like the Rac1 inhibitor NSC23766[38]. Therefore, secramine A is a unique and complementary reagent for dissecting the functions of RhoGDI1, Cdc42 and possibly other Rho GTPases. Our results underscore the usefulness of combining high-throughput synthesis, phenotypic screening, and target identification for discovering cell-permeable small molecules that perturb biological systems in unanticipated ways.

METHODS

Reagents. Secramines A, B, and C (**Supplementary Methods**) were stored as 20 mM aliquots in DMSO at –20 °C. BS-C-1 cells were obtained from ATCC. FuGENE 6 (Roche Diagnostics), cycloheximide (Calbiochem), latrunculin B (Calbiochem), brefeldin A (Calbiochem), GTPγS (Roche Diagnostics), L-α-phosphatidyl-D-*myo*-inositol-4,5-bisphosphate triammonium salt (Calbiochem), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids), L-α-phosphatidylinositol (Avanti Polar Lipids), anti-giantin (Covance Research Products), anti β-COP (Affinity Bioreagents), anti-TGN46 (Serotec), anti-myc 9E10 (Sigma), anti-Cdc42 (BD Transduction Laboratories), anti-Rac1 (BD Transduction Laboratories) and fluorescently labeled secondary antibodies (Molecular Probes) were used. D. Tortorella and H. Ploegh (Harvard Medical School) provided U373MG astrocytoma cells. J. Sondek (University of North Carolina, Chapel Hill) provided a pET28a plasmid encoding His6-tagged DH-PH domain of Dbs (623–967). G. Hoffman (Harvard University) and R. Cerione (Cornell University) provided a plasmid encoding bovine GST-RhoGDI1 and baculovirus for expression of His6-tagged Cdc42. J. Lippincott-Schwartz (US National Institutes of Health) provided a plasmid encoding

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VSVG^{ts}-KDELR[39]. The Cdc42(GDP)-RhoGDI1 complex[30], RhoGDI1[30], GST-PBD[17], VCA[14], DH-PH domain of Dbs[40], PIP2 liposomes[14] and non-prenylated Cdc42[12] were prepared as described. All the recombinant proteins, including the Cdc42-RhoGDI1 complex, were stored in 10% glycerol at –80°C. Human dynamin 2 lacking the entire proline-rich domain (amino acids 736 to 866) was cloned into pProEx HT (Invitrogen) and expressed as a His-tag fusion protein in BL21 cells. Protein expression was induced with 1 mM IPTG overnight at 18 °C at OD_{600} of 0.6. Proteins were purified with Co^{2+} beads (Talon, BD Biosciences Clontech) and by HAP columns (elution at 400 mM potassium phosphate, pH 7.2).

Transport assays and optical microscopy. VSVG^{ts}-EGFP was used to monitor exocytic transport[2] in the presence of 100 μg ml^{-1} cycloheximide and was used for the endoglycosidase H sensitivity assay[2]. NCAM-GFP and p75-GFP transport assays[27] were performed in the presence of 0.5% BSA; these proteins were retained in the Golgi by incubation at 20 °C for 30–60 min, and the cells were exposed to secramine A or vehicle for 20 min before a shift to 32 °C to activate export from the Golgi. All quantitative analyses were performed with Prism 3.0. Fluorescent images were acquired by wide-field illumination and were filtered using no-neighbors deconvolution (Slidebook, Intelligent Imaging Innovation). **Figure 6c** was filtered to enhance contrast using Adobe Photoshop.

***X. laevis* egg extract assays.** The pyrene-actin polymerization assays were performed by adding *X. laevis* egg extract[41] at 4 °C to vehicle or secramine. PIP2 liposomes or VCA polypeptide were subsequently added. The mixture was transferred to a cuvette and the fluorescence was measured at 21 °C using a Cary Eclipse instrument (Varian). PIP2 liposomes were prepared as a 48:48:4 mixture of PC:PI:PIP2 (ref. 14) by extrusion through a 0.1-μm filter (Avanti Polar Lipids). For affinity precipitation with GST-PBD[17], *X. laevis* egg extract (8 mg ml^{-1}; 200 μl of extract per condition) was added to ATP (0.2 mM) and latrunculin B (10 μM, 0.1% DMSO) at 4 °C. The mixture was then transferred to 25 °C and treated with vehicle or secramine (5 min) followed by treatment with 20 μM GTPγS and 20 μM PIP2 liposomes as indicated (10 min). GST-PBD (2.5 μM) was subsequently added. After 10 min, 150 μl of cold 2× lysis buffer (100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 400 mM NaCl, 2% Nonidet P-40, and 10% glycerol) and 50 μl of a 30% slurry of glutathione-Sepharose 4B (Pharmacia) in 2× lysis buffer were added and tumbled at 4 °C for 45 min. The mixture was then processed for western blot analysis. For experiments with recombinant nonprenylated Cdc42, *X. laevis* egg extract was treated with secramine A or vehicle (5 min), then 20 nM recombinant nonprenylated Cdc42 (GTPγS or GDP) (10 min), and then GST-PBD (10 min), followed by pulldown with glutathione-Sepharose beads. In the 'order of addition' experiment, for lane 4, components 1–3 were incubated for 10 min before the addition of secramine A. For lane 5, components 1–4 were incubated for 20 min before the addition of secramine A.

Nucleotide exchange and sedimentation assays. At $t = 0$, 10 μM [^{35}S]GTPγS (Perkin Elmer, 2,000 d.p.m. pmol^{-1}) was added to the appropriate mixtures of Cdc42, RhoGDI1, Dbs, PIP2 liposomes and EDTA in reaction buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT) at 25 °C. When needed, 1 μM RhoGDI1 was added to circumvent a partial loss in the inhibitory activity of RhoGDI1 observed upon storage of the Cdc42-RhoGDI complex. At the indicated times, a 15 μl aliquot of each reaction was diluted into 2 ml of ice-cold termination buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$) and filtered through nitrocellulose. The nitrocellulose was washed twice with termination buffer, dried and scintillation counted. PIP2 liposomes were prepared as a 65:32:3 mixture of PC:PI:PIP2 and were extruded through a 0.1-μm filter. For the PIP2 liposome sedimentation assay, components were mixed with reaction buffer to 75 μl at 25 °C; after 10 min, samples were centrifuged for 10 min at 200,000*g* and 25 °C (TL100, Beckman). The supernatant was removed, and the pellet was resuspended in 10 μl of lysis buffer. 1 μL aliquots were spotted onto nitrocellulose, blotted with an antibody against Cdc42, imaged with a LAS-3000 (Fujifilm) and quantified using Slidebook.

GTP hydrolysis assay. At $t = 0$, 20 μM [γ^{32}P]GTP (Amersham, 1,732 d.p.m. pmol^{-1}) was added to the mixture of pure components in buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT) at 25 °C. After 20 min, a 15-μl aliquot of each reaction was diluted into 0.5 ml of ice-cold activated charcoal solution (10% activated charcoal, 8% acetic acid, 2% formic acid). The solution was centrifuged for 10 min at 20,800*g*, and 250 μl of the supernatant was diluted into 0.5 ml scintillation fluid and counted. PIP2 liposomes and PC:PI liposomes were prepared as a 65:32:3 mixture of PC:PI:PIP2 and 2:1 mixture of PC:PI, respectively, by extrusion through a 0.1-μm filter (Avanti Polar Lipids).

Electron microscopy. Samples were prepared in reaction buffer, adsorbed briefly on freshly glow-discharged carbon-coated grids, negatively stained with 1.2% uranyl acetate, blot dried and imaged at 80 kV using a JEOL 100CX electron microscope[42].

Statistical analysis. All statistical analyses were performed using Prism 3.0. For **Figure 4f**, a two-tailed paired *t*-test was used; for secramine A, $P = 0.0045$; for secramine B, $P = 0.005$; for secramine C, $P = 0.6876$. For **Figure 5b**, a two-tailed unpaired *t*-test was used; for neomycin, $P = 0.0157$. For **Figure 6c**, a two-tailed paired *t*-test was used with $P = 0.0695$.

Note: Supplementary information is available on the Nature Chemical Biology website.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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Erratum: Secramine inhibits Cdc42-dependent functions in cells and Cdc42 activation *in vitro*

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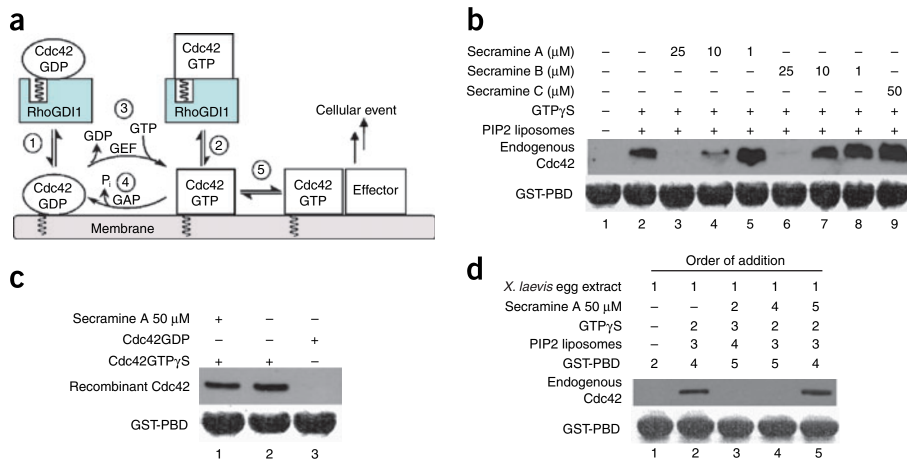
In the version of this article initially published online, two labels in Figure 3a are incorrect. The label in the square below the arrow for step 2 should read 'Cdc42 GTP' instead of 'Cdc42 GDP', and 'PI' in step 4 should be 'P_i'. These errors have been corrected in the PDF version of the article. The corrected figure is below.



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