The *Salmonella* Typhimurium effector SteC inhibits Cdc42-mediated signaling through binding to the exchange factor Cdc24 in *Saccharomyces cerevisiae*

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**ABSTRACT** Intracellular survival of *Salmonella* relies on the activity of proteins translocated into the host cell by type III secretion systems (T3SS). The protein kinase activity of the T3SS effector SteC is required for F-actin remodeling in host cells, although no SteC target has been identified so far. Here we show that expression of the N-terminal non-kinase domain of SteC down-regulates the mating and HOG pathways in *Saccharomyces cerevisiae*. Epistasis analyses using constitutively active components of these pathways indicate that SteC inhibits signaling at the level of the GTPase Cdc42. We demonstrate that SteC interacts through its N-terminal domain with the catalytic domain of Cdc24, the sole *S. cerevisiae* Cdc42 guanine nucleotide exchange factor (GEF). SteC also binds to the human Cdc24-like GEF protein Vav1. Moreover, expression of human Cdc42 suppresses growth inhibition caused by SteC. Of interest, the N-terminal SteC domain alters Cdc24 cellular localization, preventing its nuclear accumulation. These data reveal a novel functional domain within SteC, raising the possibility that this effector could also target GTPase function in mammalian cells. Our results also highlight the key role of the Cdc42 switch in yeast mating and HOG pathways and provide a new tool to study the functional consequences of Cdc24 localization.

**INTRODUCTION**

*Salmonella* are Gram-negative intracellular bacteria that cause diseases ranging from gastroenteritis to typhoid fever in humans and represent a major health problem worldwide (Ohl and Miller, 2001). These bacteria use type III secretion systems (T3SS) to deliver >30 *Salmonella* pathogenicity island (SPI)-1 and -2 effector proteins directly into the host cell cytosol (Galan and Wolf-Watz, 2006). These virulence factors enable the precise manipulation of key proteins for signaling and morphogenesis in the host. By these means, *Salmonella* cells induce their own internalization by nonphagocytic intestinal epithelial cells and are able to survive and replicate within this hostile environment. T3SS-1-delivered SPI-1 effectors are mainly required for bacterial entry into the nonphagocytic cells (Ly and Casanova, 2007). T3SS-2 is induced within host cells and delivers SPI-2-effectors important for the development of a modified phagosome called the *Salmonella*-containing vacuole (SCV), necessary for intracellular survival and favoring systemic spread (Steele-Mortimer, 2008). During systemic infection, macrophages represent an important site for replication of *Salmonella*, and T3SS-2 is essential for survival and multiplication within these immune cells (Chakravortty et al., 2002). Furthermore, effectors secreted by both T3SS are involved in the modulation of inflammatory responses (McGhie et al., 2009). Either proinflammatory or anti-inflammatory effects have been reported for different effectors, reflecting the complexity of the manipulation of host cell machinery orchestrated by this pathogen to secure its survival and proliferation.
SCV biogenesis is an extremely dynamic process involving the formation of distinct tubular networks during host cell infection through extensive changes in intracellular membrane trafficking and actin cytoskeleton (Schnoeder et al., 2011). F-actin assemblies in the vicinity of the SCV in the so-called vacuole-associated actin polymerizations, resulting in a nest or meshwork around bacteria clusters (Meresse et al., 2001). The microtubule network and molecular motors also play an essential role in the formation of the membrane tubules called Salmonella-induced filaments in a process that involves the highly conserved Rho GTase molecular switches (Bakowski et al., 2008). Rho proteins play a key role in essential physiological processes such as signal transduction, membrane trafficking, and cytoskeletal dynamics (Heasman and Ridley, 2008). Targeting of cellular Rho GTases occurs by Salmonella effectors that display guanine nucleotide exchange factor (GEF) or GTase-activating protein (GAP) activities, mimicking the activity of mammalian counterparts and resulting in the modulation of the host cellular cytoskeleton and mitogen-activated protein kinase (MAPK) pathways that operate downstream. Effector proteins that post-translationally modify either Rho GTases or their GEFs and GAPs also have been identified from distinct bacteria and shown to operate in the pathogenic process (Galán, 2009).

The ease of genetic manipulation, the availability of postgenomic tools, the wealth of biological knowledge, and the conservation of most cellular processes affected during bacterial infection in Saccharomyces cerevisiae have led to the emergence of this yeast as a very useful model for the study of bacterial virulence proteins (Rodríguez-Pachón et al., 2002; Valdivia, 2004; Aleman et al., 2005; Rodríguez-Escudero et al., 2005). S. cerevisiae has six Rho GTases, named Rho1–5 and Cdc42. Cdc42 plays a key role in the establishment of polarized growth by regulating the organization of the actin cytoskeleton. Cdc24 is the sole GEF controlling Cdc42 activity (Perez and Rincon, 2010). Moreover, Cdc24 seems to be essential for the localization of Cdc42 at the presumptive bud site on the plasma membrane and therefore for polarity establishment (Howell and Lew, 2012). This yeast also possesses five distinct signaling pathways involved in mating, pseudohyphal/invasive growth, cell wall integrity (CWI), osmoregulation, and ascospore formation, mediated by MAPKs Fus3, Kss1, Slz2, Hog1, and Smk1, respectively (Chen and Thorner, 2007). Fus3 and Kss1 are orthologues to mammalian extracellular signal-regulated kinase (ERK) 1/2, and Hog1 is orthologous to p38 (Caffrey et al., 1999). The mammalian MAPK closest to Slz2 is ERK5 (Truman et al., 2006). These MAPKs contain a highly conserved T-X-Y motif in the kinase activation loop in which both threonine and tyrosine residues must be phosphorylated to achieve kinase activation (Raman et al., 2007).

The yeast mating pathway is one of the best-understood signaling routes in eukaryotes, and its study has produced important advances in elucidating components and mechanisms participating in MAPK signaling (Dohlman and Slessareva, 2006). This pathway is activated by binding of the mating pheromone to a cell surface receptor, which causes the dissociation of the heterotrimeric G-protein into Gα subunit (Gpa1) and the Gβγ dimer (Ste4–Ste18). The GAP function of the RGS protein Sst2 promotes the reassociation of Gpa1 with Ste4–Ste18, eventually contributing to pathway adaptation and recovery. On dissociation of the G-protein, the Gβγ dimer helps to recruit the scaffold protein Ste5 to the plasma membrane, in complex with the MAPK kinase kinase (MAPKKK) Ste11, the MAPKK Ste7, and the MAPK Fus3, which phosphorylate one another in sequence. Activation of the first kinase of the cascade, Ste11, takes place at the plasma membrane through phosphorylation by its upstream activator, the PAK kinase Ste20, which in turn is activated by the small Rho GTPase Cdc42. In addition, Ste50 serves as an adaptor that links the Cdc42–Ste20 kinase complex to effector kinase Ste11. Once phosphorylated, the MAPK Fus3 translocates to the nucleus and phosphorylates Ste12, which activates transcription of pheromone response genes. The MAPK Kss1 is also transiently activated upon pheromone stimulation independently of the scaffold protein, Ste5. Some of the mating pathway components also participate in one of the two branches that lead to the activation of the MAPK Hog1. In the HOG pathway, Cdc42-dependent activation of Ste20 results in the sequential activation of the MAPKKK Ste11, the MAPKK Pbs2, and the MAPK Hog1. A number of distinct molecular mechanisms ensure that activated Ste11 does not transmit the signal to the MAPK Ste7 in response to a hyperosmotic shock, preventing spurious cross-talk between the HOG and mating pathways (Chen and Thorner, 2007).

In a previous report, we identified SteC in a genetic screen for Salmonella Typhimurium proteins that produce toxicity when over-expressed in the yeast model system (Aleman et al., 2009). This protein was shown to be translocated into host cells in a TSSS-2–dependent manner, which led to its designation as Salmonella translocated effector C (SteC; Geddes et al., 2005). SteC displays an incomplete but active catalytic protein kinase domain that is essential for SPI-2–dependent F-actin meshwork formation in host cells (Poh et al., 2008). In the interest of identifying the cellular targets of this protein, we conducted a molecular and functional characterization of SteC in the model yeast S. cerevisiae. Here we show that the N-terminal region of SteC down-regulates the yeast MAPK mating pathway at the level of Cdc42. Binding experiments indicate that SteC targets both the yeast Cdc42 GEF Cdc24 and the human Cdc24-like protein Vav1. Therefore our data point to a role for SteC in the regulation of GTase function in yeast, thereby laying the foundation for investigation of SteC activity in more complex mammalian systems.

**RESULTS**

**SteC expression down-regulates signaling through the yeast mating pathway**

SteC contains a region spanning amino acids 230–280 displaying homology to protein kinases, with the closest similarity to the human RAF proto-oncogene serine/threonine kinase (Poh et al., 2008; Aleman et al., 2009). An activated N-terminally truncated version of the MAPKKK Raf1 has been shown to substitute for Ste11 in the yeast pheromone MAPK-mediated pathway (Irie et al., 1994). With the aim of identifying the cellular targets of this protein, we used S. cerevisiae as a model to determine whether SteC was able to interfere with this pathway, by expressing SteC fused to glutathione S-transferase (GST) under the control of the inducible promoter GAL1.

Initially, we tested the effect of SteC expression on MAPK mating pathway signaling by a reporter transcription assay in which we used the pheromone-inducible FUS1 promoter and lacZ (β-galactosidase) reporter gene. As shown in Figure 1A, expression of the bacterial effector greatly reduced the transcriptional response to pheromone, suggesting a negative regulatory effect on this pathway. Next we expressed SteC in MATa Δtrc1α yeast cells, which display an autoocrine constitutive stimulation of the mating pathway due to inappropriate secretion of a-factor (Figure 1B; Ruiz et al., 2003). In this case, we analyzed the phosphorylation status of the MAPKs Fus3 and Kss1 using antibodies that detect the dually phosphorylated and thereby activated forms of MAPKs (Martin et al., 2000). We first confirmed the identity of the distinct bands detected by these antibodies by activating the mating pathway in mutants lacking the...
targeted MAPKs (Supplemental Figure S1). As observed in Figure 1B, the expression of GST-SteC resulted in a drastic reduction of the amount of phospho-Kss1 and phospho-Fus3 compared with control cells expressing GST. SteC also reduced the aberrant cell morphology of MATα itc1Δ yeast cells, which resemble the polarized mating projection of cells responding to pheromone. These results clearly indicate that SteC negatively regulates the yeast mating pathway upstream of the MAPKs.

To establish the domain of SteC responsible for this inhibitory effect on signaling, we constructed a series of plasmids expressing distinct regions of SteC as GST fusions under the GAL1 promoter (Figure 1C). Western blotting analysis confirmed that all these SteC-derived proteins were correctly expressed (Figure 1D). Galactose-dependent production of the C-terminal domain of SteC (SteC230-457) including the kinase region neither reduced the percentage of MATα itc1Δ cells displaying aberrant morphology (Figure 1C) nor resulted in a decrease of Fus3 and Kss1 phosphorylation after pheromone stimulation (Figure 1D). The shorter SteC287-457 construct, which excludes the kinase region, did not result in signaling inhibition. In contrast, expression of the N-terminal fragment of SteC, either bearing (SteC1-286) or lacking (SteC1-229) the kinase region, led to a reduced pathway output (Figure 1, C and D). A similar inhibitory effect was obtained when expressing a version of SteC in which amino acids 236–256 were deleted and thus just lacking the kinase domain. Of interest, expression of either a shorter N-terminal fragment of SteC (SteC1-156) or SteC lacking these first 156 amino acids did not alter signaling (Figure 1D). We also observed that phosphorylation of Kss1 increased its electrophoretic mobility. Accordingly, disappearance of the faster Kss1 band only occurred in samples from cells expressing full-length SteC or SteC fragments with inhibitory activity.
on the mating pathway. In sum, these data indicate that the N-terminal region spanning the first 229 amino acids is necessary for the SteC-induced inhibitory effect on signaling through the mating pathway, which is independent of SteC kinase activity.

SteC acts upstream of the mating MAPK module and independently of the Gα subunit Gpa1

Our structural analysis within the SteC N-terminal region using the SMART domain search tool identified a region encompassing amino acids 18–156 with low similarity to Regulator of G Protein Signaling (RGS) domains. Given that a receptor-coupled heterotrimeric G protein participates in the yeast mating pathway (Figure 2A), this finding suggested that SteC could act on the α subunit Gpa1 in a manner similar to the RGS protein Sst2, which down-regulates the mating pathway by promoting the GTPase activity of Gpa1 (Apanovitch et al., 1998). Consistent with this hypothesis, GST-SteC through the first 156 amino acids was able to pull down Gpa1-Myc from yeast cell extracts (Figure 2A). We next used the reporter transcription assay based on the FUS1 promoter to analyze the effect of expressing SteC in an sst2Δ mutant strain (Dohlman et al., 1995). As observed in Figure 2B, deletion of the RGS-coding gene SST2 resulted in increased sensitivity (EC₅₀) and maximum response to pheromone, as well as an increase in the basal activity of the pathway. Although overexpression of SteC reduced both basal signaling and maximum responsiveness of this mutant, it failed to restore the EC₅₀ to wild-type levels (Figure 2B), suggesting that SteC is acting by using a different mechanism than the RGS protein Sst2. Furthermore, deletion of GPA1 did not prevent the down-regulating activity of SteC on Fus3 and Kss1 phosphorylation (Figure 2C), clearly indicating that SteC acts in a Gpa1-independent manner.

To map the position in which SteC was impinging on the pheromone signaling pathway, we investigated the effect of SteC on the mating pathway. In sum, these data indicate that the N-terminal region spanning the first 229 amino acids is necessary for the SteC-induced inhibitory effect on signaling through the mating pathway, which is independent of SteC kinase activity.

FIGURE 2: SteC binds Gpa1 but acts in the mating pathway independently of both the Gα subunit and the MAPK module. (A) Left, main components that operate in the yeast mating pathway. Right, immunoblot analysis of the copurification of Gpa1-6Myc with the indicated GST-tagged SteC fragments. Transformants of the YPH499 (GPA1-6Myc) strain with the plasmids pEG(KG) (GST), pEG(KG)-SteC (SteC), pEG(KG)-SteCl-229 (1–229), pEG(KG)-SteCl1-156 (1–156), or pEG(KG)-SteCl157-457 (157–457) were grown to mid-log phase in raffinose-based selective medium and then galactose was added for a final 2% for an additional 6 h. Cell extracts (input) and GST complexes obtained by purification with glutathione-Sepharose (pull down) were analyzed by immunoblotting with anti-Myc and anti-GST antibodies. (B) Western blotting analysis of cell extracts from BY4741 (WT) and ByGpa1 (gpa1Δ) strains transformed with the empty vector pEG(KG) or pEG(KG)-SteC expressing GST (−) or GST-SteC (SteC), respectively. Cells were cultured and cell extracts analyzed as in Figure 1B. (C) Western blotting analysis of cell extracts from YPH499 (WT) cells expressing GST (−) or GST-SteC (SteC) from pEG(KG)-derived plasmids and transformed with empty vector pEG(KG)H (−) or plasmids pRS315-GAL-ST4, pEG(KG)H-cdc42G12V, and pRS425-ste11-4 in order to overproduce Ste4 or the corresponding hyperactive versions of Cdc42 and Ste11-4. In the case of cells bearing pEG(KG)-ste20ΔN, which overproduce the corresponding hyperactive version of Ste20, SteC was expressed from plasmid YCpLG-Stc-SteC. Cells were cultured and extracts were analyzed as in Figure 1B. (E) Western blotting analysis of cell extracts from YPH499 (WT) cells expressing GST (−) or GST-SteC (SteC) from pEG(KG)-derived plasmids and transformed with empty vector pRS316GAL or plasmid pGFP-G-STE5, pG-STE5-CTM, pT-G-STE11-Cpr, or pCUGF-STE5Q59L. Cells were cultured and extracts were processed and analyzed as in Figure 1B.
MAPK phosphorylation in strains in which the pathway was constitutively activated from distinct points of the signaling cascade, namely by overexpression of the G protein β subunit Ste4 (Cole et al., 1990), the GTP-bound Cdc42G12V version (Andersson et al., 2004), an N-terminally truncated version of Ste20 lacking the kinase-inhibitory CRIB domain (Ste20ΔN; Ramer and Davis, 1993; Martin et al., 1997), or the hyperactive MAPKKK version Ste11-4 (Stevenson et al., 1992). As shown in Figure 2D, SteC reduced signaling resulting from overexpression of Ste4, confirming that SteC targets elements downstream of the G protein α subunit. In contrast, SteC did not inhibit signaling in cells producing either Cdc42G12V or Ste20ΔN. These results indicate that SteC is not acting on the Ste11-Ste7-Fus3/Kss1 MAPK cascade but instead on one of the upstream components. The weak SteC-induced reduction of signaling observed when expressing Ste11-4 is compatible with this model because Ste11-4 is still regulated by phosphorylation and therefore is able to transmit signaling from upstream elements (Drogen et al., 2000). The lack of effect on the Ste20ΔN-induced activation of the pathway suggests that SteC is not directly affecting Ste20 kinase activity. On the basis of these data, SteC is likely acting on one of the early steps of the mating pathway, for example, by interfering with either the Ste5-dependent recruitment of the kinase cascade to the plasma membrane or the Cdc42-promoted Ste20 activation.

To test the first possibility, we analyzed the effect of SteC overexpression on the constitutive activation caused by artificial targeting of the Ste5 scaffold or the Ste11 MAPKKK to the plasma membrane, which occurs in a heterotrimeric G protein–independent manner. To this end, we measured MAPK phosphorylation in cells expressing Ste5G59K or Ste5-CTM versions localized at the membrane via an enhanced PM domain (Winters et al., 2005) or a foreign transmembrane domain (Pyrciak and Huntress, 1998), respectively. We also used the Ste11-Cpr version targeted to the membrane via a prenylation/palmitoylation motif (Winters et al., 2005). In all cases, overproduction of SteC led to a reduction in MAPK activation (Figure 2E), indicating that SteC is not interfering with the membrane recruitment mediated by Ste5. Furthermore, these results rule out the possibility that SteC acts on the heterotrimeric G protein.

Next we checked whether the lack of other proteins involved in the initial steps of mating signaling, like the scaffold proteins Bem1 and Far1 (Cote et al., 2011) and the Ste11 adaptor Ste50 (Truckses et al., 2006), affected the SteC-induced reduction of MAPK phosphorylation. Deletion of either BEM1 or FAR1 does not impede signaling through the pathway. However, to analyze the effect of SteC in a ste50Δ mutant, cells carried a version of Ste5 (Ste5G59K) that bypasses the requirement of Ste50 for mating signaling (Pyrciak and Huntress, 1998; Winters et al., 2005). The inhibitory effect of SteC was observed in all cases (unpublished data), indicating that Bem1, Far1, and Ste50 are not targets for SteC.

As described, SteC inhibits pathway activation by pheromone but not by the constitutively active mutant Cdc42G12V. We also determined the effect of SteC on cells expressing Cdc42G12V and treated with α-factor. Pheromone stimulation strongly increases Cdc42G12V-mediated activation of the pathway (Supplemental Figure S2). However, SteC expression was also unable to reduce MAPK phosphorylation under these conditions. These data suggest that SteC acts on Cdc42 function.

**SteC prevents cross-talk between the HOG and pheromone response MAPK pathways in hog1Δ cells**

It has been described that the MAPK Hog1 prevents cross-talk between the HOG and the pheromone response pathways (O’Rourke and Herskowitz, 1998). Thus the lack of Hog1 allows osmolarity-induced activation of the pheromone MAPK cascade in a process that is dependent on the Cdc42 node but independent of other components of the mating pathway (Figure 3A). As observed in Figure 3B, overexpression of SteC reduces the NaCl-induced phosphorylation of mating MAPKs in a hog1Δ strain, suggesting that

![FIGURE 3: SteC inhibits signaling through the Cdc42-dependent branch of the HOG pathway.](Image 311x338 to 538x735)

4434 | P. Fernandez-Piñar et al.  
Molecular Biology of the Cell
SteC is acting on a component shared by this cross-talk circuit and the mating pathway, possibly Cdc42. In accordance with these results, SteC was also able to reduce signaling through the HOG pathway in response to high salt stress, as determined by a decrease in Hog1 phosphorylation (Figure 3C). This inhibitory effect of the bacterial effector was magnified in a strain lacking Ssk2/22. In this case one of the two inputs to the HOG pathway is missing, leaving only the HOG branch that is mediated by Cdc42 (Figure 3C). Taken together, these data strongly support the idea that SteC acts as a negative regulator of Cdc42-mediated signaling.

Cdc42 overexpression rescues SteC-induced inhibition of yeast growth

We previously showed that overexpression of either full-length or the N-terminal region of SteC in yeast cells leads to strong growth inhibition (Aleman et al., 2009). Therefore overexpression of the yeast SteC target is expected to relieve the lack of growth induced by this bacterial effector. Therefore, as a parallel and complementary approach to the epistasis analysis, we performed a genetic screen searching for suppressors of SteC toxicity. To this end, we transformed a GAL1-driven yeast cDNA library in cells overexpressing SteC under the same promoter and selected clones able to grow on galactose-inducing conditions. All positive clones shared the same DNA insert corresponding to yeast CDC42 (Figure 4A). Human Cdc42 is known to functionally replace its yeast orthologue (Munemitsu et al., 1990). Accordingly, overexpression from a different GAL1-based plasmid of either yeast or human Cdc42 also overcame SteC toxicity (Figure 4A). Moreover, overexpression of yeast proteins related to Cdc42 function, such as Cdc43, Gic2, Rho3, Rsr1, and Ste20, which have been shown to compensate growth defects of distinct defective alleles of Cdc42 (Kozinski et al., 2000, 2003), also alleviated SteC toxicity to a distinct extent (Supplemental Figure S3). Taken together, these results confirm that SteC-induced growth defects are caused by inhibition of Cdc42 function and are in agreement with the observed effects on the mating pathway.

To carry out cellular localization studies, we tagged SteC with green fluorescent protein (GFP). Fluorescence microscopy observations revealed that SteC-GFP predominantly showed peripheral localization, likely associated to the plasma membrane, but also colocalized with the marker FM4-64 that stains vacuolar and endosome membranes (Vida and Emr, 1995; Figure 4B). SteC1-229 presented the same localization as the full-length SteC, whereas SteC157-457 showed a cytoplasmic distribution in the cells (Figure 4C), indicating that the N-terminal part of the protein is responsible for membrane targeting. GFP-Cdc42 also localized associated to membranes either in the presence or the absence of SteC (Figure 4C). Moreover, we analyzed the presence of phosphoinositides phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate in yeast cells overexpressing SteC. These lipids are important for recruitment of proteins that contain phosphoinositide-binding domains (Strahl and Thorner, 2007) and play a role in Cdc42 functionality (Yakir-Tamang and Gerst, 2009). SteC expression did not alter the cellular distribution of these phosphoinositides, as detected by using specific fluorescent probes (Stefan et al., 2002; Supplemental Figure S4). These data ruled out that mislocalization of Cdc42 or phosphoinositides alteration is the cause of the Cdc42 functional defect induced by SteC.

SteC physically interacts with Cdc24, the sole GEF for Cdc42

To determine whether SteC binds Cdc42, we carried out copurification experiments with yeast cells coexpressing GST-SteC and either yeast or human hemagglutinin (HA)-tagged versions of Cdc42. The negative-constituent HsCdc42177N (Feig, 1999) and the constitutively active HsCdc42G12V versions were also tested for their ability to bind SteC. No interaction was detected between SteC and any of these Cdc42 mutant proteins (Figure 5A and unpublished data). In contrast, the Salmonella mutant effector SopB9568, known to interact with Cdc42 (Rodriguez-Escudero et al., 2011), pulled down human Cdc42 (Figure 5A). Both yeast two-hybrid and in vitro copurification assays with recombinant proteins expressed in Escherichia coli were also negative for interaction between SteC and Cdc42 (unpublished data).

Activation of yeast Cdc42 relies on its sole GEF protein, Cdc24. This GEF controls Cdc42 function, and therefore cdc24 mutants display the same phenotype as loss-of-function cdc24 mutant cells
region of SteC interacts with Cdc24, likely resulting in the inhibition of Cdc42 function.

SteC binds to Schizosaccharomyces pombe and human Cdc24 homologues

The use of the yeast model led us to identify Cdc24 as a target of SteC. Then we wanted to know whether SteC was able to bind similar GEFs from other organisms, including human, the natural host of Salmonella. We first tested interaction with Schizosaccharomyces pombe Scd1, the Cdc24 GEF homologue to Cdc24 (Perez and Rincon, 2010), by copurification experiments using a HA-tagged version of this protein. As shown in Figure 6A, E. coli-produced recombinant GST-SteC was able to pull down HA-Scd1 from S. pombe extracts. Therefore the ability of SteC to bind Cdc24 GEFs is extended to evolutionary distant yeast like the fission yeast S. pombe. Of interest, no binding of SteC to Rom1, a GEF for S. cerevisiae Rho1 GTPase, was observed (unpublished data). This is consistent with the lack of inhibitory effect of SteC on the cell integrity MAPK pathway mediated by Rho1 (Aleman et al., 2009).

Like other Dbl-family GEFs (Rossman et al., 2005), S. cerevisiae Cdc24 has a catalytic Dbl homology (DH) domain responsible for the GEF activity. The fact that sequence similarity between Cdc24 and Scd1 mainly occurs within their DH domains (31% identity) led us to examine whether SteC interacted with this domain of Cdc24. To this end, we looked at the ability of GST-SteC to pull down the DH domain of Cdc24 (Cdc24^{287-479}) tagged to Myc. As shown in Figure 6B, both full-length Cdc24-Myc and Cdc24 (DH)-Myc copurified with GST-SteC. These results indicate that the SteC Salmonella effector is likely to target GEFs through binding to the catalytic DH domain.

Next we tested the interaction of SteC with Vav1, one of the members of the human Dbl family of Rho GEFs that displays significant similarity at the catalytic DH domain to yeast Cdc24 (24% identity). Vav1 activates Cdc42, Rac1, and RhoA GTPases and is involved in key biological functions, including actin cytoskeleton reorganization and activation of ERK and Jun N-terminal kinase, as well as development and activation of immune cells (Lazer and Katzav, 2011). Copurification in vitro assays with recombinant E. coli-produced proteins showed that both GST-SteC and GST-Cdc42, used as a positive control, were able to bind the polyhistidine (polyHis)-tagged DH domain of the human GEF Vav1 (Figure 6C). Moreover, the N-terminal fragment of SteC (SteC^{1-229}) also pulled down the DH domain of Vav1. These results suggest that SteC binding to the DH domain of GEFs might interfere with the corresponding Rho GTPase function in Salmonella-infected cells. Although the most plausible mechanism underlying this effect would be the inhibition of the catalytic activity of the GEF, in vitro assays showed that neither GST-SteC nor GST-SteC^{1-229} altered the exchange ability of Vav1 toward Rac1, the GTPase to which Vav1 GEF activity is most effective (Rapley et al., 2008; Figure 6D).

SteC prevents nuclear localization of Cdc24

Our results suggested that SteC does not inhibit the catalytic activity of bound GEF. To investigate how this effector could be altering GEF function, we analyzed several known layers of regulation of Cdc24, namely phosphorylation, oligomerization, and spatial regulation. The PAK kinase Cla4 has been shown to phosphorylate Cdc24, leading to a mobility shift in SDS–PAGE (Guili et al., 2000; Bose et al., 2001). As shown in Figure 7A, SteC does not inhibit phosphorylation of Cdc24 caused by the expression of a constitutively active version of Cla4 (Martin et al., 1997).
It is also known that Cdc24 shuttles between the nucleus and sites of cell growth. Cdc24 accumulates in the nucleus during late M phase, the mother–daughter bud neck during M phase (Toenjes et al., 1999). To investigate whether SteC affects the localization of this GEF, we examined GFP-Cdc24 localization in cells expressing GST, GST-SteC, or GST-SteC\(^{1-229}\). As expected, Cdc24 localized to the nucleus in unbudded and small-budded control cells after 6 h of galactose-induced expression of GST (Figure 7B). Remarkably, the expression of GST-SteC clearly reduced the percentage of cells with nuclear Cdc24 (to 15%, down from 32% in control GST-expressing cells). Nuclear Cdc24 was reduced further to just 1% of cells expressing the SteC N-terminal domain (Figure 7C). Western blotting analysis revealed that the amount of GFP-Cdc24 was similar in cells expressing either GST or SteC-derived GST fusion proteins (Figure 7D), indicating that the alteration of Cdc24 nuclear localization was not due to degradation of the protein. Oligomerization regulates subcellular localization of Cdc24 (Mionnet et al., 2008). As observed in Figure 7E, however, SteC did not alter the amount of Cdc24-HA bound to Myc-Cdc24 in yeast communoprecipitation experiments, ruling out the inhibition of Cdc24 oligomerization as the mechanism underlying the observed localization defects.

As expected, after 6 h of galactose induction, SteC- or SteC\(^{1-229}\)-expressing cells became big and round, and thus it was not possible to follow Cdc24 localization at different cell-cycle stages. To overcome this limitation, we carried out a Cdc24 localization analysis with cells after just 2 h of galactose-induced expression of GST or SteC-derived GST-fusion proteins, in which GFP-Cdc24 production was confirmed by Western blotting (Figure 7H). Consistent with data presented in Figure 7C, whereas a high percentage of control cells expressing GST showed Cdc24 in the nucleus at G1 or M phase, a reduction of nuclear Cdc24 was observed in cells expressing SteC (Figure 7I). Expression of SteC\(^{1-229}\) completely prevented nuclear Cdc24 in cells at these stages (Figure 7F and G). In addition, the percentage of cells displaying Cdc24 localization at polarized growth sites was also reduced upon expression of either SteC or SteC\(^{1-229}\), although this effect was milder than that observed for nuclear Cdc24 localization (Figure 7G). Taken together, these results indicate that Cdc24 localization is significantly altered by SteC expression.

**DISCUSSION**

More than 20 *Salmonella* proteins are known to be translocated by the T3SS-2 across the phagosomal membrane (Srikanth et al., 2011).
However, the specific roles of many of these effectors are far from being well understood. Unveiling their functions is hindered by the fact that bacterial effectors are often multifunctional proteins with different activities able to target many processes of eukaryotic physiology. Moreover, different effectors may display overlapping effects and cooperate to promote specific alterations in the host cell (Galan, 2009). Such redundancy could account for the absence of virulence attenuation in animal models by bacteria lacking a specific effector, as occurs with SteC (Geddes et al., 2005; Poh et al., 2008). These constraints have prompted the use of alternative approaches to investigate the functional roles of secreted bacterial proteins.

Here, by exploiting the yeast model, we identified a novel activity of SteC as a negative regulator of the GTPase Cdc42. Several observations support this conclusion: 1) overexpression of SteC specifically inhibits Cdc42-mediated MAPK pathways, 2) epistasis analysis places SteC at the level of Cdc42 in the mating pathway, 3) overexpression of either *S. cerevisiae* or human Cdc42 is able to counteract yeast growth inhibition caused by SteC, 4) this growth rescue is also achieved by overexpressing yeast suppressor genes of cdc42 mutations, and 5) SteC displays a similar localization as Cdc42 in the yeast cell. A large body of work has shown that various bacterial effectors modulate the GTPase cycle either by acting as GEFs or GAPs (Orchard and Alto, 2012).

**FIGURE 7:** SteC modifies Cdc24 localization and does not affect Cdc24 phosphorylation and oligomerization. (A) Western blotting analysis of cell extracts from YPH499 cells harboring plasmids p414-3xmycCdc24F1 and pEG(KG) (–) or pEG(KG)-cla4ΔN (Cla4ΔN) and cotransformed with pEG(KG)H (–) or pEG(KG)-SteC (SteC). Cells were grown to mid-log phase in raffinose-based selective medium, and then galactose was added for a final 2% for an additional 6 h. The 3xmycCdc24 was detected with 9E10 antibodies, and anti-G6PDH was used to detect G6PDH as a loading control. (B) Fluorescence microscopy photographs showing in vivo localization of Cdc24 and colocalization with 4′,6-diamidino-2-phenylindole staining. YKT725 cells expressing GFP-tagged Cdc24 and transformed with pEG(KG) (GST), pEG(KG)-SteC (GST-SteC), or pEG(KG)-SteCΔ1-229 (GST-SteCΔ1-229) were cultured as in A. Samples were taken at 6 h for fluorescence microscopy analysis. Bars, 5 μm. (C) Percentage of cells from the same cultures used in B, showing nuclear Cdc24. (D) Western blotting analysis of GFP-Cdc24 from the same cultures used in B, using anti-GFP and anti-GST antibodies. (E) RAY1773 cells expressing HA-tagged Cdc24 were cotransformed with p414-3xmycCdc24F1 plasmid and pEG(KG)-SteC (GST-SteC) or pEG(KG)H-SteC (GST-SteC) and cultured as in B. HA-tagged Cdc24 was immunoprecipitated from cell extracts, and bound Myc-Cdc24 was assayed by Western blotting. (F) Fluorescence microscopy photographs showing the in vivo localization of YKT725 cells cultured as in B but with samples taken at 2 h after galactose addition. Bars, 5 μm. (G) Percentage of cells showing nuclear (gray bars) or growth sites (bud tips or septa, white bars) localization of GFP-Cdc24 at different stages of the cell cycle. The indicated transformants were cultured as in F. n > 100 cells for each strain and for each cell cycle stage. (H) Western blotting analysis of YKT725 cell extracts of cells showed in G.
or by modifying the activity of GTPase regulators, as reported for the Rho GEF inhibitor EspH from enteropathogenic and enterohemorrhagic E. coli (Dong et al., 2010). In the case of SteC, our data indicate that Cdc42 inhibition is likely exerted via binding to its cognate GEF. First, overexpression of SteC in yeast cannot suppress the effect of the constitutively GTP bound mutant Cdc42G12V. Second, SteC is able to interact both in vivo and in vitro with Cdc24, the sole GEF for this GTPase in yeast, but not with Cdc42. Third, SteC also binds to the Cdc24-like GEFs S. pombe Scd1 and human Vav1. Fourth, cellular localization of Cdc24 is altered by SteC expression.

The effector EspH has been shown to bind directly the DH domain of multiple Rho GEFs, thus preventing their binding to Rho and thereby inhibiting nucleotide exchange–mediated Rho activation (Dong et al., 2010). SteC also interacts with the DH domain of GEFs, but it is not able to inhibit in vitro the exchange capacity of Vav1, a human GEF that belongs to the Dbl family and contains a catalytic DH domain similar to yeast Cdc24. Although it is possible that our in vitro assays lacked an essential cofactor required in cells to exert the biological effect, the molecular mechanism responsible for the action of SteC on the target GEF seems to be different from that described for EspH. Inhibition of the exchange activity is not the only way to modulate GEF function in eukaryotic cells. For example, MLK3 binds to the Rho activator p63RhoGEF, impeding its activation by Goq and thus preventing Rho activation (Swenson-Fields et al., 2008). Our results suggest that alteration of Cdc24 cellular localization could account for the defect in Cdc24 functionality. Cdc24 shuttling between the nucleus and the cytoplasm regulates cell polarity (Nern and Arkowitz, 2000; Shimada et al., 2000) due to its role in localizing Cdc42 at growth sites (Howell and Lew, 2012). Far1 has been shown to be responsible for nuclear sequestration of Cdc24 (Nern and Arkowitz, 2000; Shimada et al., 2000). In addition, the GTPase Rsr1 seems to be involved not only in Cdc24 targeting to the growth site, but also in its activation. However, the fact that the double far1Δ rsr1Δ mutant is viable suggests that additional proteins are also involved in recruiting Cdc24 to specific sites at the cell cortex (Shimada et al., 2004). Binding to SteC might inhibit the interaction of Cdc24 with any of these proteins involved in proper localization of active Cdc24. This hypothesis is consistent with our data showing that Rsr1 overexpression partially recovers the growth inhibition imposed by SteC, bearing in mind that overexpression of Rsr1 rescues the growth defect of a cdc24 mutant unable to interact with Rsr1 (Shimada et al., 2004). It is also tempting to speculate that SteC could modify Vav1 localization in human cells, since this GEF has also been shown to undergo nucleocytoplasmic redistribution (Adam et al., 2000; Bertagnolo et al., 2012). Additional experiments in mammalian cells are needed to test this possibility.

Expression in yeast provides a convenient way to define the functional domains of bacterial effector proteins (Aleman et al., 2005). According to our results, the N-terminal region of SteC is responsible for the observed Cdc42 inhibition. The C-terminal, Ral1-homologous, kinase domain of SteC is necessary for inducing the formation of the F-actin meshwork around the Salmonella-containing vacuole inside host cells (Poh et al., 2008). Therefore our findings suggest that SteC, as with many other bacterial effectors (Dean, 2011), is composed of functionally distinct domains conforming to a modular protein. Salmonella Typhimurium SipA effector is cleaved by caspase-3 within the host cell, thereby generating two independent active domains (Srikanth et al., 2010). These authors also reported that other caspase-3 sites identified within Salmonella Typhimurium proteins appear to be restricted to T3SS translocated effectors, which indicates that proteolytic cleavage may be a strategy used by the pathogen for activating effectors delivered in a precursor form. Of interest, we found a putative caspase-3 recognition site, DXKD (spanning amino acids 205–208), located between the two functional regions of SteC. It is therefore tempting to speculate that this protein could be processed by proteolytic cleavage into two functionally independent effector peptides.

We identified SteC in a screen for Salmonella proteins that cause toxicity in yeast cells. Overexpression of SteC resulted in large, swollen, and un budded cells with depolarized actin (Aleman et al., 2009), resembling the phenotype described for cdc42 or cdc24 mutant cells (Perez and Rincon, 2010) and consistent with the Cdc42-inhibitory function of SteC described here. Thus our results suggest that not only the kinase domain, but also the N-terminal region of SteC could be modulating the actin cytoskeleton via Cdc42. This idea is in agreement with previous observations by Poh et al. (2008), who found that full-length SteC induced formation of F-actin clusters and cables randomly distributed throughout the cytoplasm, whereas in mammalian cells expressing the kinase domain alone, these structures were mainly found at the cell periphery. Because the Rho family of small GTPases comprises important regulators of actin organization (Hall, 1998), targeting these GTPases or their regulators is one of the most common mechanisms used by bacterial effectors to manipulate cytoskeleton pathways. The mammalian GEF Vav1 has been shown to regulate actin cytoskeleton changes associated with RhoA, Rac1, and Cdc42 (Olson et al., 1996). Therefore binding of SteC to Vav1 suggests that this Salmonella effector could be influencing actin dynamics via Vav1. In addition, Vav1 has been reported to play a role in bacterial lipopolysaccharide-mediated macrophage activation and nitric oxide synthase production (Godambe et al., 2004). The action of SteC on Vav1 would be also consistent with the function of the Salmonella SPI-2 in countering the nitrosative stress in host macrophages (Das et al., 2009).

By expressing SteC in yeast, we have been able to evaluate the effect of Cdc42 function on yeast MAPK signaling. As opposed to the inhibition of the mating pathway described here, we previously reported that expression of SteC led to the activation of Slit2, the MAPK operating in the CWI pathway (Aleman et al., 2009). It is likely that the morphogenetic and cell wall alterations originating from SteC-induced Cdc42 inhibition leads to a stress sensed by the CWI pathway. These results confirm that Cdc42 is not directly mediating signal transduction through the CWI pathway and underscores the role of this GTPase as a key component of the mating pathway. Because the CWI pathway is mediated by the GTPase Rho1 (Chen and Thorner, 2007), our results also point to a specificity of SteC for Cdc42 GEFs in the yeast model. Consistently, we could not detect binding of SteC to the Rho1 GEF Rom1 (Perez and Rincon, 2010). Although this GEF shares 23% identity with Cdc42 within the DH domain, other regions distinct from the DH domain of Rom1 could restrict its binding to SteC, in contrast to that occurring for Cdc42. We also show here that signaling induced by pheromone can be reduced to basal levels by interfering with the Cdc42 GEF Cdc24, emphasizing its key role not only in the morphogenetic changes associated with mating, but also in signaling through the MAPK mating pathway. Moreover, signaling inhibition caused by SteC supports the model in which two complementary signaling nodes—the Ste5-bound kinases and the Cdc42–Ste20 complex—assemble at the yeast plasma membrane to initiate activation of the mating pathway (Winters et al., 2005). More broadly, our results show that expression of bacterial effectors in yeast cells can be exploited to uncover novel properties of these proteins, as well as to provide new tools to study yeast signaling.
**Strain** | **Genotype** | **Source**
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YPH499 | MATa ade2-101 trp1-63 leu2-1 ura3-52 his3-D200 lys2-801 | P. Hieter (British Columbia University, Vancouver, Canada)
YDM400 | MATa ade2-101 trp1-63 leu2-1 ura3-52 his3-D200 lys2-801 sst2-Δ2 | H. Dohlman (North Carolina University, Chapel Hill, NC)
BY4741 | MATa his3Δ1 leu2Δ met15Δ ura3Δ | EUROSCARF
Y04500 | MATa his3Δ1 leu2Δ met15Δ ura3Δ, itc1::kanMX4 | EUROSCARF
Y01268 | BY4741 isogenic, far1::kanMX4 | EUROSCARF
Y02724 | BY4741 isogenic hog1::kanMX4 | EUROSCARF
Y03340 | BY4741 isogenic, ste50::kanMX4 | EUROSCARF
Y03439 | BY4741 isogenic, bem1::kanMX4 | EUROSCARF
Y03857 | BY4741 isogenic ste7::kanMX4 | EUROSCARF
Y00993 | BY4741 isogenic slt2::kanMX4 | EUROSCARF
Y06981 | BY4741 isogenic kss1::kanMX4 | EUROSCARF
Y03042 | BY4741 isogenic fus3::kanMX4 | EUROSCARF
TM141 | MATa leu2 ura3 trp1 his3 | Alonso-Monge et al. (2001)
TM257 | MATa ura3 leu2 trp1 his3 ssk2::LEU2 ssk22::LEU2 | Raitt et al. (2000)
BYgpa1 | BY4741 isogenic gpa1::kanMX4 | H. Martín (Complutense University, Madrid, Spain)
RAY1773 | MATa leu2-3,-112 ura3-52 trp1-1 ade2-1 his3-11 can1-100 GAL bar1 cdc24X3HA::Klactis URA | Mionnet et al. (2008)
YKT725 | MATa cdc24α::kanMX6 ura3-52 his3Δ-200 trp1α-63 leu2Δ-1 lys2-801 pKT1154 (pRS415-Cdc24p-GFP-CDC24) | Fujimura-Kamada et al. (2012)

**TABLE 1:** *S. cerevisiae* strains used in this work.

**MATERIALS AND METHODS**

**Bacterial and yeast strains and yeast genetics methods**

The *S. cerevisiae* strains used in this study are listed in Table 1. The *Salmonella* Typhimurium SL1344 strain (Hoiseth and Stocker, 1981) was used in this study was kindly provided by F. Garcia del Portillo. The PPG103 *S. pombe* strain (h- leu1-32 ura4-D18) was provided by Pilar Pérez (Moreno et al., 1991). Standard procedures were used for yeast genetic manipulations (Sherman, 1991). Yeast transformations were performed with the lithium acetate method (Ito et al., 1983).

**Culture conditions**

YPD (1% yeast extract, 2% peptone, and 2% glucose) broth or agar was the complete medium used for growing the yeast strains. Synthetic medium (SD) contained 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, and 2% glucose and was supplemented with appropriate amino acids and nucleic acid bases (Sherman, 1991). SG and SR were SD with 2% glucose and was supplemented with appropriate amino acids and ammonium sulfate, 0.5% ammonium sulfate, and 2% glucose.

**DNA manipulation and plasmids**

General DNA methods were used, using standard techniques (Sambrook et al., 1989). The steC-containing plasmids used in this study are described in Supplemental Table S1 and were constructed by PCR amplification from *Salmonella* Typhimurium SL1344 genomic DNA, using oligonucleotides described in Supplemental Table S2 and cloning in the correct orientation into the corresponding site of pEG(KG) (URA3 GAL1-GST leu2-d 2μ; Mitchell et al., 1993). Amplified DNA was verified by DNA sequencing.

**Screening for suppressors of SteC toxicity**

YPH499 strain bearing plasmid YCpLG-SteC for SteC expression driven by GAL1 promoter was transformed with a yeast CDNA library in pYES2 plasmid (kindly provided by Enrique Herrero; Espinet et al., 1995) and plated on SG medium in order to select clones able to grow in the presence of galactose as carbon source and therefore able to suppress the impaired growth of SteC-expressing cells. From 128,000 clones screened, 20 were selected by this means. Twelve of them showed dependence on the pYES2-based plasmid for growing on galactose, as revealed by plasmid loss and 5-fluoroorotic acid counterselection.

**Quantitative β-galactosidase assays**

β-Galactosidase assays were performed as described (Hao et al., 2003). Briefly, cells were transformed with plasmid pRS423-FUS1-β-gal for expression of β-galactosidase under the control of FUS1 promoter as reporter for mating pathway activation. Overnight cultures were refreshed in SD medium, grown during the day, refreshed in SR medium at an OD600 of 0.01, and cultured overnight. Then they were refreshed in the same medium at an OD600 of 0.3, and galactose was added to a final concentration of 2%. After 3–4 h (OD600 of 0.6–0.8), 90 μl of each culture was transferred to a 96-well plate and α-factor was added to different final concentrations (0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, and 30 μM) in a final
volume of 100 μl. Plates were incubated at 30 ºC for 90 min, and 20 μl of FDG solution (130 mM 1,4-piperazinediethanesulfonic acid, pH 7.2, 0.25% Triton X-100, 0.5 mM fluorescein-di-β-galactopyranoside) was added. Plates were incubated at 37ºC for 1 h, and reaction was stopped by adding 20 μl of NaHCO₃, 1 M, before measuring the fluorescence signal in a PerkinElmer fluorimeter (PerkinElmer, Waltham, MA).

Preparation of yeast extracts and immunoblot analysis
The procedures used have been previously described (Martin et al., 2000). Immunodetection of actin and Myc-tagged proteins was carried out using monoclonal C4 (IMP Biomedicals, Santa Ana, CA) and 9E10 (Covance, Berkeley, CA) antibodies, respectively. Anti–phospho-p44/42 MAPK (Thr-202/Tyr-204), anti–phospho-p38 MAPK (Thr-180/Tyr-182; Cell Signaling, Beverly, MA), anti–GST (Santa Cruz Biotechnology, Santa Cruz, CA), anti–TAP (Open Biosystems, Huntsville, AL), anti-His (Sigma-Aldrich, St. Louis, MO), anti–glucose-6-phosphate dehydrogenase (G6PDH) antibodies (Sigma-Aldrich), anti–GST J1-8 (Clontech, Mountain View, CA) and anti–HA (Roche, Indianapolis, IN) antibodies were also used. The primary antibodies were detected either using a horseradish peroxidase–conjugated secondary antibody with the ECL detection system (Amersham, Piscataway, NJ) or a fluorescently conjugated secondary antibody with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Expression of recombinant proteins in E. coli and preparation of extracts
Recombinant GST- or histidine-tagged proteins were expressed from pGEX-KG or in E. coli strain Rosetta DE3 (Novagen, Gibbstown, NJ). Cells were collected and lysed by sonication in phosphate-buffered saline (PBS) buffer containing 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (DTT), 1 mM EDTA, and 1 mg/ml lysozyme in the presence of protease inhibitor cocktail (Roche). Extracts were clarified by centrifugation and then stored at −80ºC.

Copurification assays
For in vivo binding assays, yeast cells were resuspended in lysis buffer lacking SDS and Triton X-100 and broken with glass beads in a Fast Prep machine. Samples were adjusted to a final volume of 200 μl. Then, 100 μl of 50% slurry of glutathione-Sepharose 4B resin was added and incubated on a roller for 3–12 h at 4°C. Beads, recovered by brief centrifugation, were then washed five times in lysis buffer and subsequently resuspended in 30 μl of 2× SDS–PAGE sample loading buffer. Bound proteins were eluted by boiling for 10 min. After centrifugation at 13,200 rpm for 15 s, the resulting supernatant was resolved by SDS–PAGE, transferred to nitrocellulose, and processed for immunoblotting. For in vivo binding assays to Qpa1, yeast cells were resuspended in 250 μl of the corresponding lysis buffer (40 mM triethanolamine, pH 7.2, 2 mM EDTA, 150 mM NaCl, 2 mM DTT, 3 mM MgCl₂, and protease inhibitors) and broken with glass beads in a Fast Prep machine. Lysates were cleared by centrifugation at 3200 rpm twice for 10 min each, and 1.5% Triton X-100 was added to the resulting supernatant solution for 1 h. Then the insoluble material was removed by centrifugation at 13,200 rpm for 10 min. Samples were then processed in the same way as described previously.

In vitro binding assays were performed by binding GST or GST-fused proteins from E. coli extracts to glutathione-Sepharose 4B resin, and then either E. coli or yeast extracts bearing the corresponding tagged proteins were added and processed as described.

Immunoprecipitation assays
To analyze Cdc24 oligomerization, RAY1773 yeast cells expressing HA-tagged Cdc24 transformed with p414-3xmycCdc24Fl plasmid for expression of myc-tagged Cdc24 and pEG(KG)H, pEG(KG)H-SteC, or pEG(KG)H-SteC were lysed in the same buffer used in copurification assays. Dynabeads PanMouse immunoglobulin G (Invitrogen, Carlsbad, CA), 25 μl/sample, was incubated with anti-HA.11 antibody (clone 16B12; Covance) at a dilution of 1:150 for 2–3 h at 4°C. After washing with PBS and 0.1% bovine serum albumin, cell extracts were added and the mixture was incubated for 3–4 h. Then, beads were washed three times, eluted by boiling in 30 μl of 2× SDS–PAGE sample loading buffer, and assayed by Western blotting with anti-myc and anti-HA antibodies.

Guanine nucleotide exchange assays
In vitro guanine nucleotide exchange was carried out essentially as described previously (Rojas et al., 2003). For loading reactions, 400 nM Rac1 and 80 nM Bodipy FL GDP were incubated in 1 ml of buffer consisting of 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 5% (vol/vol) glycerol, 2 mM DTT, and 0.08% (vol/vol) NP-40, and fluorescence ($\lambda_{ex} = 502$ nm; $\lambda_{em} = 511$ nm) was monitored with a PerkinElmer LS55 spectrophotofluorometer (slits, 2.5 nm) thermostatted to 15°C. At the indicated time, 4 μl of a stock solution consisting of 1.25 μM Vav1 (DH/PH/CRD fragment; Chrencik et al., 2008) either alone or preincubated for 5 min with 12.5 μM SteC, was added, resulting in final concentrations of 5 nM Vav1 and 50 nM SteC. Unloading reactions were carried out similarly, except that Rac1 was preloaded with Bodipy FL GDP and incubated with 20 μM GDP before additions of Vav1 and SteC, resulting in final concentrations of 10 and 100 nM, respectively.

Microscopy and immunofluorescence
Fluorescence microscopy of live yeast cells for GFP detection was performed as described (Rodriguez-Escudero et al., 2006). Cells harboring GFP fusion vectors with MET25 promoter were cultured overnight in selective SD medium and then washed three times with sterile water and refreshed in the same medium lacking methionine. After 6 h at 30°C cell suspensions were spotted on slides for microscopy observation. For FM4-64 staining, cells were pelleted, resuspended in the same medium containing 40 μM FM4-64, and incubated for 1 h at 30°C. Differential interference contrast (DIC) images were acquired by using an Eclipse TE2000U microscope (Nikon, Melville, NY) fitted with an Orca C4742-95-12ER digital camera (Hamamatsu, Hamamatsu, Japan) and then processed by using Aquacosmos Imaging System software (Hamamatsu).

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