Fission Yeast Ras1 Effector Scd1 Interacts With the Spindle and Affects Its Proper Formation

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ABSTRACT

Ras1 GTPase is the *Schizosaccharomyces pombe* homolog of the mammalian Ha-Ras proto-oncoprotein. Ras1 interacts with Scd1 (aka Ral1), a presumptive guanine nucleotide exchange factor for Cdc42sp, to control organization of the cytoskeleton. In this study, we demonstrated that the *scd1* deletion (*scd1*Δ) induced hypersensitivity to microtubule destabilizing drugs and instability of the minichromosome. Overexpression of *scd1* induced formation of abnormal spindles and chromosome missegregation. The *scd1* deletion worsened the defects of spindle formation in tubulin mutants; by contrast, it did not induce lethality in mutants defective in the spindle pole bodies. These genetic data suggest that Scd1 can interact with tubulin with substantial specificity to affect proper spindle formation and chromosome segregation. Subcellular localization data further illustrated that a GFP-Scd1 fusion protein can associate with the spindle. Finally, we showed that unlike *ras1*Δ and *scd1*Δ, *byr2*Δ (affecting the Ras1 effector for mating) is not synthetically lethal with the tubulin mutations. These data collectively suggest that the Ras1 pathway can impinge upon microtubules through Scd1, but not Byr2, to affect proper spindle formation and chromosome segregation.

RAS G-proteins are generally known as the switches for signal transduction (Barbacid 1990). Ras cycles between the GDP-bound biologically inactive state and the GTP-bound active state, a process catalyzed by guanine nucleotide exchange factors (GEFs). Mutant forms of Ras proteins that are predominantly GTP bound are oncogenic.

The mechanism by which hyperactive Ras can lead to cancer is only partially understood. Mammalian Ras has at least two well-established downstream pathways. Ras activates the conserved Raf-MAP kinase cascade (Van Aelst *et al.* 1993; Vojtek *et al.* 1993), which in turn can activate gene expression, *i.e.*, that of cyclin (Filmus *et al.* 1994), to affect cell division. Ras also induces reorganization of the cytoskeleton, which requires Rho GTPases, such as Cdc42Hs and Rac (reviewed by Van Aelst and D'Souza-Schorey 1997). The relevance of the cytoskeleton organization to tumorigenesis remains an open question.

Our laboratory is using the fission yeast, *Schizosaccharomyces pombe*, as the model system to study the function of Ras. *S. pombe* contains one known Ras homolog, Ras1 (Fukui *et al.* 1986; Nadin-Davis *et al.* 1986). Like mammalian Ras, Ras1 in *S. pombe* has at least two downstream pathways, which appear to regulate two distinct functions: mating and cytoskeleton organization. This is supported by the fact that the *ras1* null (Δ) mutants are sterile and have abnormally round cell morphology.

We and others have shown that the Byr2 protein kinase is the Ras1 effector for controlling mating (Wang *et al.* 1991; Chang *et al.* 1994). Byr2 is functionally analogous to Raf, in that they both control a MAP kinase cascade, which ultimately affects gene expression (Xu *et al.* 1994). To regulate the organization of the cytoskeleton, Ras1 interacts with Scd1 (aka Ral1; Fukui and Yamamoto 1988), which is a putative GEF for Cdc42sp (Chang *et al.* 1994); Cdc42sp in turn regulates Shk1 (aka Pak1 and Orb2; Marcus *et al.* 1995; Ottilie *et al.* 1995; Verde *et al.* 1998), which is a member of the conserved p21-activated protein kinase family (PAK). We have shown that Ras1 can enhance the physical interaction between Scd1 and Cdc42sp in the yeast two-hybrid system (Chang *et al.* 1994). We have also identified another regulator for Scd1, Scd2 (Chang *et al.* 1994; aka Ral3, Fukui and Yamamoto 1988). Our data suggest that Scd2 acts as a scaffold to strengthen the physical interactions between Scd1 and Cdc42p and between Cdc42sp and Shk1, which ultimately leads to a more efficient activation of Shk1 *in vivo* (Chang *et al.* 1994, 1999).

Although we have begun to understand the complex protein-protein interactions in the Ras1-Cdc42sp pathway, we still know very little about how Ras1 affects the organization of the cytoskeleton and how this contributes to our understanding of tumor development. We shed some light on this in a previous study, in which we characterized a conserved molecule, Moe1, that was

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isolated from a yeast two-hybrid screen using Scd1 as bait (Chen *et al.* 1999). Our data suggest that one of the key functions of Moe1 is to negatively affect microtubule stability or assembly or both. Microtubules in *moe1*Δ cells have aberrant morphologies and are abnormally stable and abundant. Interestingly, *moe1*Δ is synthetically lethal in combination with a loss of function in the Ras1-Cdc42sp pathway, producing double mutants that are defective in proper spindle formation. These double mutant cells either are blocked in prophase or lose viability in the cold due to chromosome missegregation. Chromosome missegregation is analogous to genome instability that is frequently observed in tumors. Since Moe1 is a potential microtubule regulatory protein, we postulate that Scd1 may affect spindle formation by impinging upon the microtubule cytoskeleton.

In this study, we present evidence demonstrating that Scd1 can genetically interact with tubulins with substantial specificity to affect proper spindle formation. More important, Scd1 can associate with the spindle in the cell. Finally, we show that the ability to affect proper spindle formation is a common property of the Ras1-Scd1 pathway, but not the Ras1-Byr2 pathway.

MATERIALS AND METHODS

Parental strains: The generic wild-type strain in our laboratory is SP870 (*h*^{90}, *ade6.210*, *leu1.32*, *ura4-D18*). Strains SPSCD1U, SPM3, SPSCD2L, SPM2, and SPRN were all derived from SP870 to contain the *scd1* deletion (*scd1::ura4*), the *scd1* mutation (*scd1-1*), the *scd2* deletion (*scd2::LEU2*), the *scd2-1* mutation, and the *ras1* deletion (*ras1::ura4*::pUC119), respectively (Chang *et al.* 1994). Mutant strains containing the *nda2-KM52* and *nda3-KM311* mutations (Toda *et al.* 1983) and the parental wild-type strain (*h*$^{-}$, *leu1.32*) are from Paul Nurse. For clarification we named the wild-type strain from the Nurse lab PN1. The profilin (*cdc3-313*), *cut11-1*, *sad1-1*, and calmodulin (*camE14*) mutants are from K. Gould (Balasubramanian *et al.* 1994), R. McIntosh (Strain 76; West *et al.* 1998), I. Hagan (Strain 274; Hagan and Yanagida 1995), and T. Davis (Strain MP64; Moser *et al.* 1997), respectively. Strain 318 containing a Cut11 tagged with green fluorescent protein (GFP) at its C terminus is also from R. McIntosh (West *et al.* 1998). Strain YP10.22 containing a linear minichromosome is from U. Fleig (Fleig *et al.* 1996).

Microbial manipulation: The rich medium used was YEAU, which is YEA (Alfa *et al.* 1993) supplemented with 75 mg/liter uracil, and the minimum medium was minimal medium (MM) with appropriate supplements (Alfa *et al.* 1993). Thiabendazole (TBZ) and benomyl were dissolved in DMSO as stocks, and control plates lacking these drugs contained the same amount of DMSO. Cells transformed with plasmids containing the *nmt1* promoters were seeded on MM plates containing 20 μM thiamine to achieve maximal repression of the *nmt1* promoter. A nitrogen-free MM medium was prepared by eliminating NH_4Cl. To synchronize cells in G1 by starvation (Goshima *et al.* 1999), cells were pregrown to early log phase ($2–5 \times 10^6$ cells/ml), washed, and incubated in the nitrogen-free MM medium overnight at 30°. Cells were examined microscopically to determine the synchronization efficiency. Hydroxyurea (HU, 11 mM; Moser *et al.* 1997) was added to cells in early log phase in YEAU at 30° for 6 hr. HU blocks DNA synthesis but allows cells to "grow" by apical extension without mitosis. In our study, after a 6-hr incubation in HU, F-actin dots appear at both ends of the tested cells, which suggests that these cells had passed new end takeoff (NETO; Mitchison and Nurse 1985) and were arrested in interphase. HU was removed by centrifugation, and cells were shifted to 23° in fresh medium and examined over time. Cell viability was determined by plating on MM medium at 32°.

Plasmid constructions: pSCD1L and pALLT17N were created by swapping the *ura4* in pSCD1U and pALUT17N (Chang *et al.* 1994) with *LEU2*. To express GFP (GFP[S65T]) fusion proteins, a *Pst*I/*Bam*HI fragment of the *gfp* gene (Heim *et al.* 1995) was created by PCR to replace the cMYC tag in pARTCM (Chang *et al.* 1994). The resulting vector was named pALG. *scd1* was excised from pGAD-SCD1 (Chang *et al.* 1994) and cloned into pALG to create pALG-SCD1. A DNA fragment containing the coding sequence of HA1-tagged Scd1 was released from pALUSCD1 (Chang *et al.* 1994) and cloned into pREP1 (Basi *et al.* 1993) to construct pREP1-HASCD1. The *nmt1* promoter in pREP1 has been reported to be six to seven times more powerful than the *adh1* promoter (Basi *et al.* 1993). The integration plasmid pIAG-SCD1B was created by inserting a *Sph*I-*Bam*HI fragment from pALG-SCD1 containing the *adh1* promoter fused with *gfp-scd1* and a *Hin*dIII fragment of *ura4* into pUC119.

Strain constructions: To knock out *scd1*, *scd2*, and *ras1*, pSCD1L, pSCD2L, and ras1▽leuHd/pUC7 were used (Nadin-Davis *et al.* 1986; Chang *et al.* 1994), respectively; *byr2* was deleted as described (Wang *et al.* 1991). pALLT17N was used to overexpress the dominant negative form of Cdc42sp. pIAG-Scd1B was linearized at the *Xho*I site in the *scd1* coding region before transforming the *scd1* mutant SPM3. The resulting strain was named ECP26. To create *scd1*Δ *cam-E14* and *scd1*Δ *cut11-1* double mutants, protoplast fusion was carried out between strain SPSCD1U and strain MP64 and between SPSCD1U and strain 76, followed by tetrad analysis.

Stability of minichromosome: Gene deletion was carried out in the tester strain YP10.22, which turns red in the indicator medium because it contains the *ade6-210* mutation. The *ade6-210* mutation is complemented by *ade6-216*, carried by the linear minichromosome (Niwa *et al.* 1989). Thus, the loss of minichromosome causes YP10.22 cells to turn red in the indicator medium. To examine the loss of minichromosome, cells were spread on the indicator plates (MM plus 5 mg/liter adenine); from each strain, 12 colonies of equal size were mixed and ~5000 cells were spread on the indicator plates. The percentage of red colonies that emerged afterward is reported.

Cell permeabilization: The protocol is as described (Masuda and Shibata 1996). Briefly, cells in early log phase were digested with zymolyase 80T (1 mg/ml; ICN) for 0.5–1 hr at 30°, washed with MESS [0.1 M MES, pH 6.5, 5 mM EDTA, 1 mM spermidine, 0.5 mM spermine, 1 M Sorbitol, 20% DMSO, 0.1 mM Trolox, 5 mM DTT, 0.2 mM PMSF, and a protease inhibitor cocktail from Sigma (St. Louis)], and permeabilized in the same buffer containing 0.5% Triton X-100 for 7 min on ice.

Fluorescence microscopy: The general procedures for immunostaining and for calcofluor and 4′,6-diamidino-2-phenylindole (DAPI) staining are as described (Alfa *et al.* 1993). To visualize microtubules, TAT1 (1:5, overnight; Woods *et al.* 1989) was used as the primary antibody and FITC- or TRITC-conjugated anti-mouse IgG (Sigma; 1:50; 6 hr) was the secondary antibody. Cells containing pALG-SCD1 were examined as soon as colonies appeared after transformation to avoid saturating cells with high levels of GFP-Scd1. GFP-Scd1 signals reduced markedly after standard fixation; therefore, we fixed these samples in 0.025% glutaraldehyde for 3 min first and then in 3.5% formaldehyde for 20 min prior to microtubule

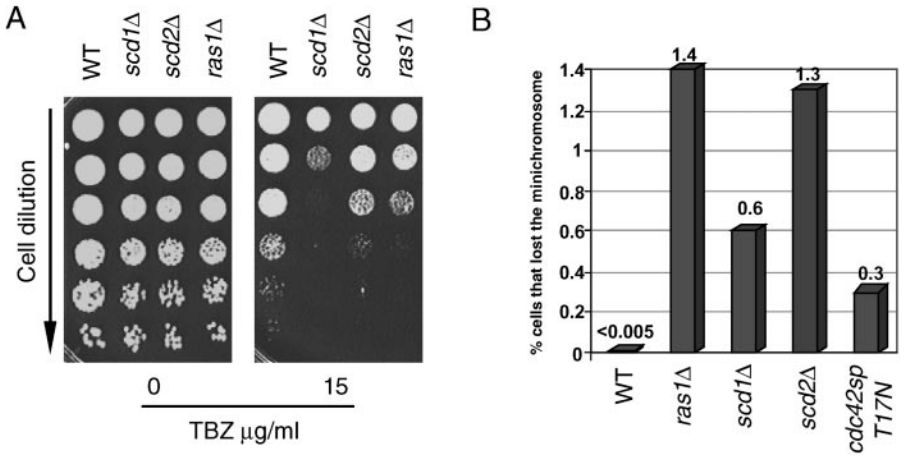

FIGURE 1.—TBZ sensitivity and minichromosome instability. (A) Approximately the same number of cells were serially diluted 1:5 and spotted on YEAU plates containing indicated concentrations of TBZ. Plates were incubated at 32° for 3 days. The relevant genotype of each strain is labeled on top. The strains used were SP870 (WT), SPSCD1U (*scd1*Δ), SPSCD2L (*scd2*Δ), and SPRN (*ras1*Δ). (B) Gene deletions or the presence of *cdc42spT17N*, encoding a dominant negative form of Cdc42, are indicated at the bottom. The numbers on top of each column indicate the percentage of cells that lost the minichromosome.

staining. The endogenous Scd1 is expressed at a very low level and its subcellular localization patterns seem vulnerable to fixation. We used a PCR-based method (BÄHLER *et al.* 1998a) to tag endogenous Scd1 with GFP and 13 × cMYC. We were unable to detect GFP-tagged Scd1, and the 13 × cMYC-tagged Scd1 appeared scattered and diffused in the cell after immunostaining. Under conditions where GFP-Scd1 was clearly visible on the spindle in live cells (*i.e.*, those containing the integrated Scd1-GFP), it became undetectable after immunostaining using anti-GFP antibodies [CLONTECH (Palo Alto, CA) and BÄHLER *et al.* 1998b].

Examining Scd1 localization in various mutant strains: pALG-SCD1 was linearized by *Blp*I to allow *adh1-gfp-scd1* to integrate into the *ars1* loci in the following cells: a *ras1*Δ strain (SPRN), the *scd2-1* mutant strain (SPM2), a *moe1*Δ strain (MOE1U; CHEN *et al.* 1999), and a *shk1*Δ strain kept alive by an integrated *nmt1-shk1* (GILBRETH *et al.* 1998). We tested Scd1 localization in the latter strain in the presence of 20 μM thiamine. Under this condition the cells are round, indicative of partial loss of *shk1* function. These mutant strains were also transformed with pALG-SCD1 to better determine whether Scd1 localization to the cell tips and equator can be altered in these cells. Strain ECP26 was transformed by pALLT17N, which expresses Cdc42sp[T17N]. The detection of GFP-Scd1 is as described above.

RESULTS

Loss of function in *scd1* induces TBZ sensitivity and minichromosome instability: To investigate whether a loss of function in the Ras1-Cdc42sp pathway affects microtubule functioning, we first examined whether deleting *scd1* rendered cells hypersensitive to microtubule-destabilizing drugs, using a colony formation assay. As shown in Figure 1A, *scd1*Δ cells were indeed hypersensitive to TBZ. Under such treatment, *scd1*Δ cells frequently contained missegregated chromosomes (by DAPI staining; data not shown). Similar results were obtained using another microtubule poison, benomyl. To test whether *scd1*Δ affected proper chromosome separation, we measured the stability of a linear minichromosome. As shown in Figure 1B, the minichromosome was lost at a markedly high frequency in *scd1*Δ cells (0.6%); in contrast, its loss in wild-type cells was not detectable (<0.005%).

There are at least two interpretations of our results. It is possible that *scd1*Δ affects a mitotic checkpoint such that these cells divided prematurely before the spindle was properly constructed and/or attached to the kinetochore. As an alternative, *scd1*Δ may affect the functioning of the spindle. To examine the former, we deleted *scd1* in a β-tubulin mutant (carrying the *nda3-KM311* mutation; HIRAOKA *et al.* 1984). This β-tubulin mutant contains microtubules that are too unstable at 18° to allow for efficient spindle formation. Therefore, at 18° a spindle-assembly checkpoint is activated to block cell division. We reasoned that if *scd1*Δ bypasses this checkpoint, one would expect the Scd1 β-tubulin double mutant to go on dividing at 18°, which can be easily detected by the presence of a septum. As shown in Figure 2A, the Scd1 β-tubulin double mutant and the β-tubulin single mutant failed to septate at 18° and were arrested with condensed undivided chromosomes (data not shown). In addition, *scd1*Δ cells, just like wild-type cells, could be arrested at metaphase by overexpressing Mad2 (Figure 2B), a spindle checkpoint inducer (HE *et al.* 1997), suggesting that the Mad2-dependent spindle checkpoint was unaffected by *scd1*Δ. On the basis of these results, we conclude that *scd1*Δ is unlikely to alter known spindle checkpoint(s).

The *scd1* deletion worsens the defect of spindle formation in the tubulin mutants: Next, we investigated whether Scd1 can interact with tubulins to affect proper spindle formation. We reasoned that if Scd1 interacts with tubulins, *scd1*Δ would worsen the phenotype of tubulin mutants, such as the β-tubulin mutant, as described above. In addition, we examined an α1-tubulin mutant (carrying the *nda2-KM52*; TODA *et al.* 1984), which, like the β-tubulin mutant, was unable to generate a spindle at 18°. As shown in Figure 3A, while *scd1*Δ and the single tubulin mutants each grew at 23°, the Scd1 α1-tubulin and Scd1 β-tubulin double mutants failed to grow. These data suggest that *scd1*Δ worsens the growth defects of both the α1-tubulin and β-tubulin mutants.

We analyzed how the Scd1 α1-tubulin double mutant passed through mitosis at 23° to learn the cause of the

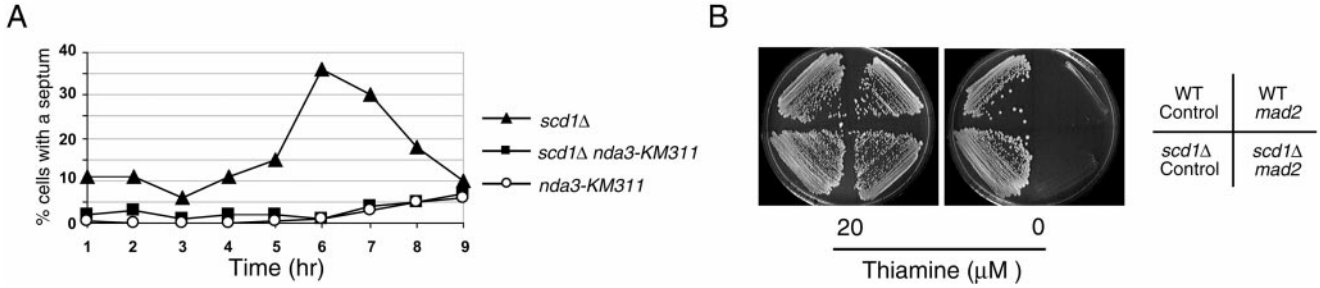

FIGURE 2.—*scd1*Δ does not bypass the spindle checkpoint. (A) Cells were synchronized in the G1 phase by nitrogen starvation and then allowed to grow in fresh rich medium for 4 hr at 30°. Afterward, they were shifted to 18°, and time points were taken and fixed briefly with formaldehyde. The percentage of cells with a septum was scored after calcofluor staining. The β-tubulin cold-sensitive mutant carries the *nda3-KM311* mutation, and *scd1* was deleted in this strain as well as in its parental wild-type strain (PN1). (B) Overexpression of *mad2* was controlled by the thiamine-repressible *nmt1* promoter (pREP3X-MAD2; HE *et al.* 1997). Fresh transformants were streaked on MM plates with or without thiamine at 32°. Strains tested were SP870 (WT) and SPSCD1U (*scd1*Δ).

observed growth defect. Various strains were first synchronized in S-phase (by HU) at 30° and then allowed to progress through mitosis at 23°. Over time, the percentages of mitotic cells (those with condensed chromosomes) with and without a detectable spindle were measured and plotted in Figure 3B. Furthermore, the same set of data was replotted in Figure 3C to show the relative abundance of mitotic cells with a spindle. Together, these results indicate that although both the α1-tubulin single mutant and the Scd1 α1-tubulin double mutant entered mitosis at about the same time and contained approximately the same percentage of cells in M-phase (Figure 3B), almost three times more of the mitotic Scd1 α1-tubulin mutant cells were without a spindle (Figure 3C). Moreover, in most of the mitotic Scd1 α1-tubulin mutant cells, the only detectable microtubule signal was a dot near the nucleus (Figure 3B, right, arrowhead). Note that we also examined cells that were free floating to make sure that we were not looking down at the end of a short spindle. These results support the hypothesis that the deletion of *scd1* further cripples the ability of the tubulin mutant to form a functional spindle.

To examine the terminal phenotype of the double mutant, the cultures of both the single and double mutants were maintained at 23° for 24 hr. The α1-tubulin mutant cells were still in log phase and almost all viable (95%). In comparison, the Scd1 α1-tubulin double mutant lost viability (<50% viable), which correlates with an accumulation of aberrant cells containing missegregated chromosomes (Figure 3D, groups I–IV). We examined an asynchronous culture of the Scd1 α1-tubulin double mutant at 23° and found deformed cells indistinguishable from those shown in Figure 3D, indicating that this anomaly is not an artifact of the HU treatment.

We also investigated whether *scd1*Δ is synthetically lethal with mutations affecting the spindle pole bodies (SPB), which are the fungal microtubule organizing centers for nucleating the spindle. We deleted *scd1* in three mutants carrying the temperature-sensitive *cut11-1*, *sad1-1*, and *camE14* mutations. The wild-type alleles of all three genes encode proteins that localize to the SPBs, and all mutants fail to make a normal spindle and die of chromosome missegregation at the nonpermissive temperature. We found no obvious synthetic lethality in the resulting double mutants; *i.e.*, the colony sizes of all double mutants were the same as those seen in the single mutants at all temperatures examined (data not shown). In conclusion, our data indicate that Scd1 can interact with tubulins with substantial specificity to affect spindle formation.

Scd1 associates with the spindle in the cell: Since Scd1 can genetically interact with tubulins, we went on to investigate whether Scd1 physically associates with microtubules in the cell. A DNA construct expressing a GFP Scd1 fusion protein (GFP-Scd1) under the control of the *adh1* promoter was integrated into the chromosome of a *scd1* mutant strain. The resulting strain is fertile and elongated in cell morphology and divides without obvious abnormalities in spindle formation. These observations suggest that GFP-Scd1 is biologically functional. As shown in Figure 4A, GFP-Scd1 seemed to concentrate between the two mitotic nuclei, as one would expect if Scd1 associates with the spindle, while the GFP control diffused throughout the cell (CHEN *et al.* 1999 and Figure 4, B and C). We did not detect GFP-Scd1 associated with interphase microtubules.

Since the yeast nuclear membrane does not break down during mitosis, is it possible that the internuclear GFP-Scd1 signal was from Scd1 attached to the nuclear membrane? To investigate this possibility, we permeabilized live cells with a detergent to dissolve the nuclear membrane and to remove soluble proteins from the cell. To monitor the efficiency of permeabilization, we examined Cut-11 tagged with GFP (Cut11-GFP), which associates with the nuclear envelope (WEST *et al.* 1998 and Figure 4A). Our data show that the internuclear signal of GFP-Scd1 was far more resistant to permeabili-

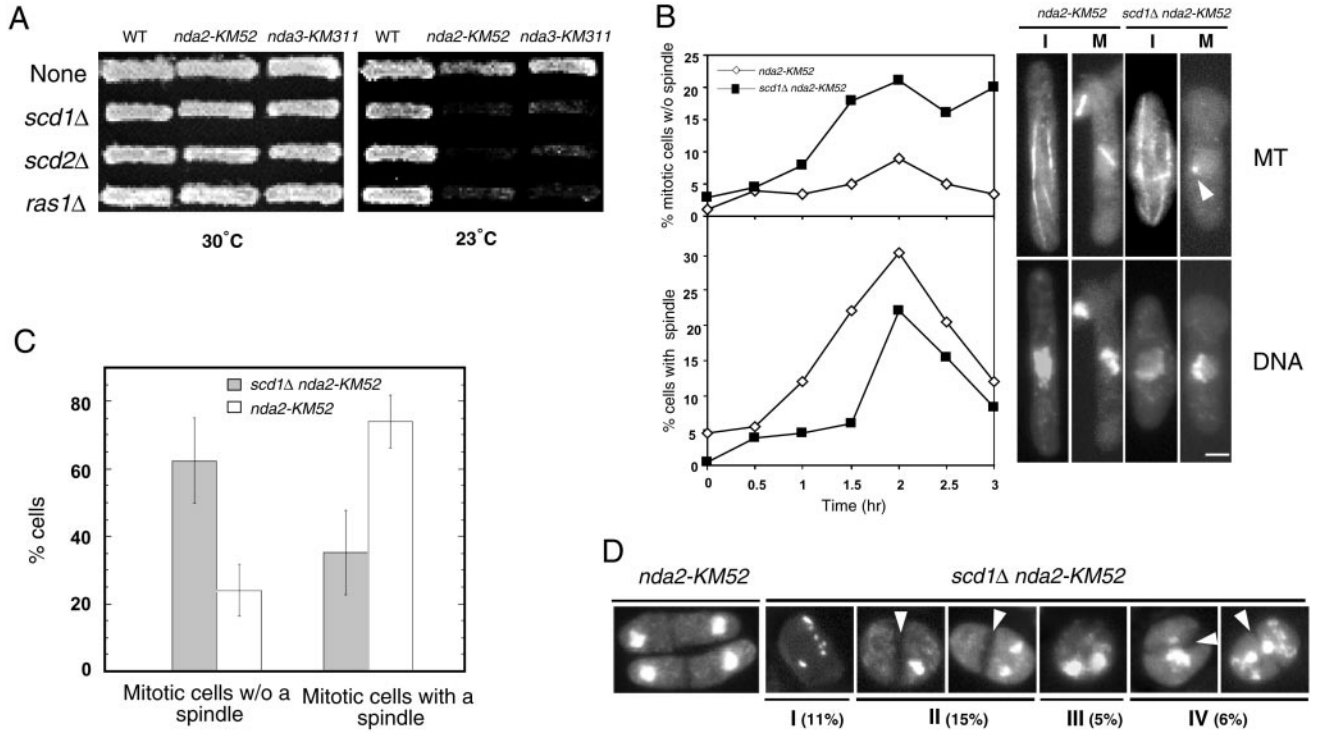

FIGURE 3.—The loss of function in the Ras1 pathway worsens the defects of spindle formation in the tubulin mutants. (A) The components lacking in the α1- and β-tubulin mutants (*nda2-KM52* and *nda3-KM311*, respectively) are indicated on the left. The wild-type strain used was PN1. Cell patches were generated by replica plating on rich medium at 30° for 2 days or at 23° for 3 days. (B) The α1-tubulin mutant (*nda2-KM52*) and Scd1 α1-tubulin double mutant (*scd1*Δ *nda2-KM52*) were synchronized by HU and then released into fresh liquid medium at 23°. Time points were taken and stained for microtubules and DNA. On the left, the percentages of mitotic cells with and without a spindle were plotted over time. On the right, cells were stained for microtubules (MT) and DNA. I, Interphase; M, M-phase. An arrowhead marks the only detectable microtubule signal in an abnormal mitotic cell. Bar, 3 μm. (C) Among mitotic cells, the percentages of those with and without a spindle from all the time points in B were averaged to show the relative abundance of mitotic cells with a spindle in the Scd1 α1-tubulin double mutant. Error bars indicate standard deviation ($n = 7$). (D) Aberrant morphology of synchronized cells, as described in B, after 24 hr at 23°. Cells were fixed in 4% formaldehyde and stained with DAPI. We grouped these abnormal cells into four groups: (I) cells with fragmented or undetectable chromosomes; (II) cells with unequal numbers of nuclei between two "daughter cells"; (III) binucleate cells without a spindle (microtubule staining is not shown); (IV) cells with at least one of the nuclei "cut" by the septum. The position of the septum is indicated by an arrowhead. The frequency at which each group of cells appeared is indicated at the bottom.

zation than that of Cut11-GFP; moreover, the spindle-like pattern of Scd1 seemed more readily observable after clearing away GFP-Scd1 from the cytosol and nucleus. Hence, it is unlikely that the internuclear signal of Scd1 is associated with the nuclear membrane.

Next we investigated whether the internuclear "spindle-like" GFP-Scd1 was indeed associated with microtubules. After cells were treated with either TBZ or cold shock (on ice)—both of which induce microtubule depolymerization (MATA and NURSE 1997)—their internuclear GFP-Scd1 signal readily disappeared. In contrast, GFP-Scd1 remained internuclear in a profilin mutant (*cdc3-313*; BALASUBRAMANIAN *et al.* 1994) that was kept at its nonpermissive temperature to allow for F-actin disorganization (data not shown). Additionally, we counterstained fixed cells with anti-tubulin antibody and showed that the GFP-Scd1 signal overlapped with the spindle (Figure 4B, columns 2 and 3).

As reported previously, GFP-Scd1 was also detected in the nucleus (CHEN *et al.* 1999). Here we found that most of the nuclear Scd1 can be removed by permeabilization, but some Scd1 is resistant to permeabilization and appears as five to six dots (Figure 4A, the third panel from the left). The significance of this is not clear. Additionally, when GFP-Scd1 was overexpressed from a high-copy plasmid, it was readily detectable at the cell ends in interphase cells and at the cell equator during late anaphase (Figure 4C, columns 2 and 3). The significance of this observation is discussed later.

Scd1 localization is altered by a dominant interfering Cdc42sp: Scd1 is part of a protein complex that also includes Ras1, Scd2, Cdc42, Moe1, and Shk1, and we showed that these proteins interact in a cooperative fashion. Therefore, we investigated whether proper Scd1 localization requires other components in the same protein complex. We found that a loss of function in *ras1*, *scd2*, *moe1*, or *shk1* did not detectably affect the localization of GFP-Scd1 (MATERIALS AND METH-

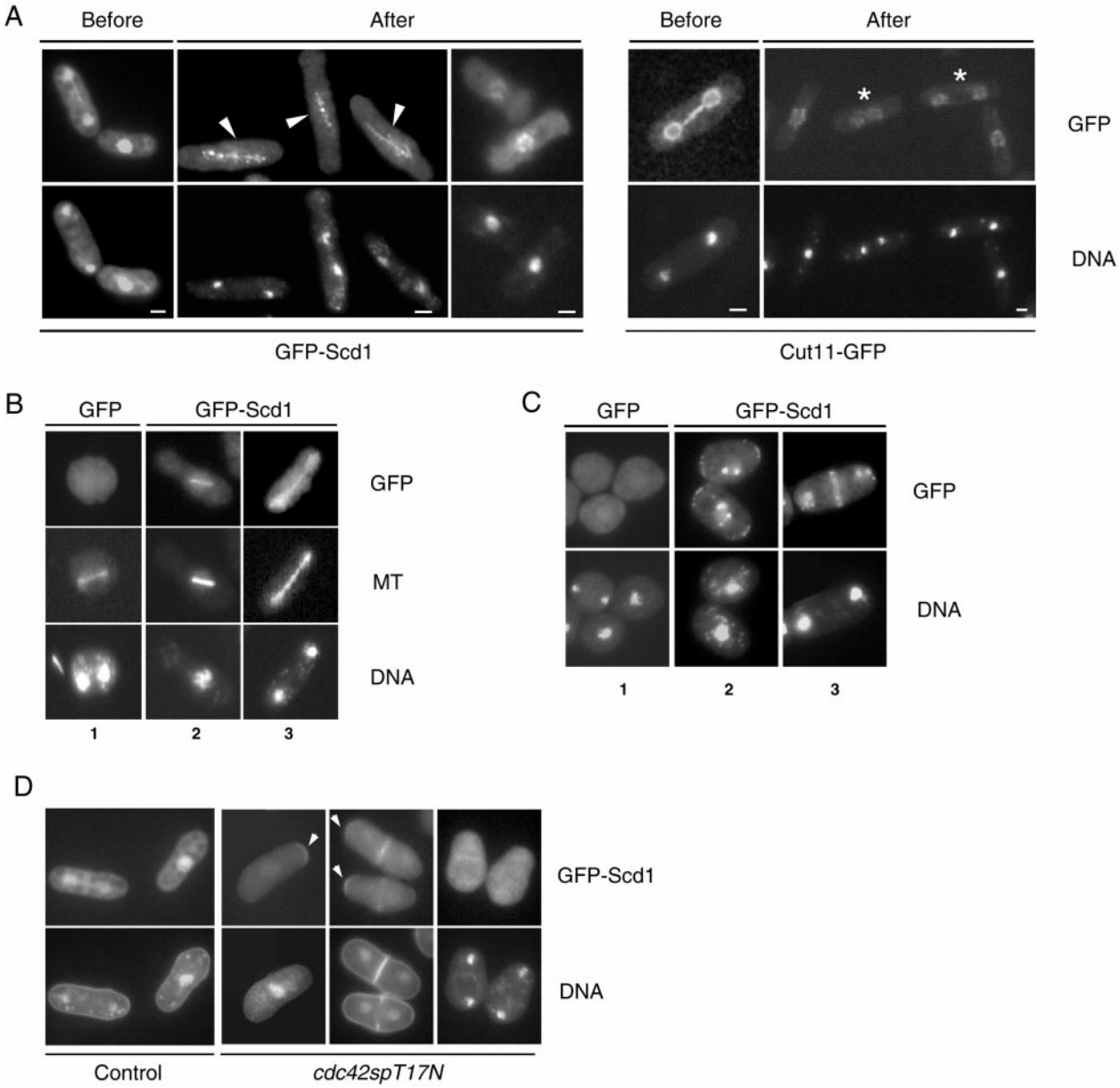

FIGURE 4.—GFP-Scd1 localization in the cell. (A) Strain ECP26 carrying an integrated *adh-gfp-scd1* before and after permeabilization is shown to the left, while cells carrying Cut11-GFP are shown to the right. Arrowheads mark cells containing GFP-Scd1 that resembled a spindle. Asterisks mark anaphase cells carrying Cut11-GFP that had lost the internuclear GFP signal. Before the detergent wash, the internuclear Cut11-GFP was readily detected in >65% of the anaphase cells, but it fell to 5% after permeabilization; in contrast, the internuclear signal of GFP-Scd1 decreased only slightly from 60 to 40%. We note that the Cut11-GFP signal did not diminish completely after permeabilization; a fuzzy and punctuated pattern could still be detected around the nucleus. Bars, 3 μm. (B and C) *scd1*Δ cells (strain SPSCD1U) were transformed with either the control GFP vector (pALG, column 1) or a vector expressing GFP-Scd1 (pALGSCD1). Cells in B were fixed and immunostained to reveal microtubules (MT), while cells in C were alive. (D) Strain ECP26 (*adh-gfp-scd1*) was transformed by either a control plasmid or a plasmid expressing *cdc42sp[T17N]*. To observe a higher percentage of cells in the same stage of the cell cycle, the resulting cells were synchronized by HU. Arrowheads mark Scd1 signal that is readily observable at the cell ends.

ods). In particular, Scd1 remained associated with the spindle in the majority of the anaphase cells. However, in the presence of a dominant interfering Cdc42sp, Cdc42sp[T17N], the levels of Scd1 were markedly reduced from the nucleus and the spindle (Figure 4D). Intriguingly, more Scd1 was readily detectable at the cell ends, which was usually difficult to detect unless Scd1 is overexpressed from a high-copy plasmid.

Scd1 overexpression induces spindle abnormalities and chromosome missegregation: We and others have examined microtubules in *scd1*Δ cells by immunostaining (Pichová *et al.* 1995; Chen *et al.* 1999). Although the microtubule cytoskeleton in *scd1*Δ cells has an abnormal appearance, no obvious structural defects in the spindle (*i.e.*, the formation of a V or star-shaped spindle) or in chromosome segregation can be detected. However, we

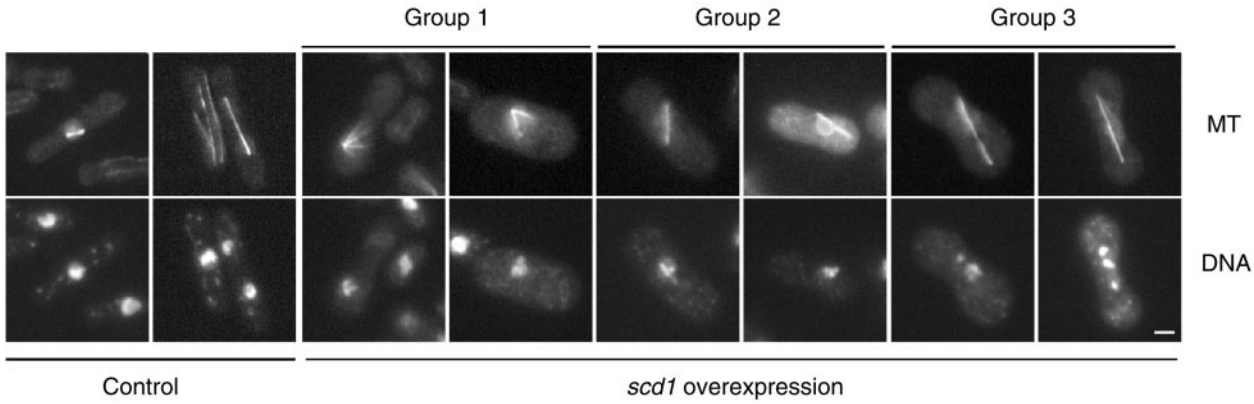

FIGURE 5.—Scd1 overexpression causes spindle abnormality and chromosome missegregation. Wild-type strain PN1 was transformed with pREP1-HASCD1 or a control vector (pREP1), and the transformed cells were cultured in thiamine-free medium for 26 hr before being stained to view microtubules (MT) and DNA. Bar, 3 μm.

found that overexpressing Scd1 in wild-type (*scd1*$^+$) cells from a high-copy plasmid containing the strongest thiamine-repressible *nmt1* promoter (pREP1-HASCD1) was capable of causing numerous mitotic defects.

Wild-type cells carrying the pREP1-HASCD1 plasmid were grown without thiamine for 26 hr to allow for accumulation of Scd1 and then immunostained to view the spindle (Figure 5). We found that about half of the mitotic cells displayed abnormalities that can be categorized into three groups: (1) 40% of them had a V or star-shaped spindle with a condensed chromosome; (2) 33% showed an anaphase spindle (>3 μm) but the sister-chromatids were undivided; and (3) 27% had lagging or unevenly distributed DNA along the anaphase spindle. The viability of these cells dropped 25% from $t = 0$. By contrast, we did not detect abnormal mitosis or loss of viability in cells carrying either a control plasmid (Figure 5) or the same Scd1 plasmid whose *nmt1* promoter was shut off (data not shown). On the basis of these results, we speculate that overexpression of Scd1 titrates out Scd1 binding proteins that are critical for spindle functioning and chromosome disjunction.

Ras1 mediates chromosome segregation by acting through Scd1, but not Byr2: Ras1 has two known effectors, Scd1 and Byr2. We asked whether Ras1 influences chromosome segregation by acting through the Scd1-Cdc42sp pathway or Byr2 or both. Our data show that a loss of function in other components in the Ras1-Cdc42sp pathway also induced hypersensitivity to TBZ (*i.e.*, *ras1*Δ and *scd2*Δ, Figure 1A, and a *shk1* mutation; S. MARCUS, personal communication) and the loss of a minichromosome (*i.e.*, *ras1*Δ, *scd2*Δ, and the presence of *cdc42sp[T17N]*, Figure 1B). Furthermore, *ras1*Δ and *scd2*Δ (Figure 3A) or expression of *cdc42sp[T17N]* (data not shown) induced lethality in the α1- and β-tubulin mutants, and the terminal phenotypes of these double mutant cells at 23° were indistinguishable from those in Figure 3D. These results suggest that these cells also died of abnormal chromosome segregation. In contrast, *byr2*Δ did not induce any synthetic lethality in the tubulin mutant (data not shown). We conclude that the ability to affect spindle functioning is a common feature specific for the Ras1/Scd1 but not for the Ras1/Byr2 pathway.

DISCUSSION

Our results demonstrate a novel function for the Ras1 effector Scd1 in *S. pombe*, namely, the ability to physically associate with the spindle and affect its formation and/or function. We showed that *scd1*Δ induces TBZ and benomyl hypersensitivity and minichromosome instability. We further illustrated that *scd1*Δ worsens the defects of spindle formation in both α1- and β-tubulin mutants, but not in mutants defective in SPBs, and our subcellular localization data suggest that Scd1 associates with the spindle in the cell. Furthermore, overexpression of Scd1 in wild-type cells induces abnormalities in spindle formation and chromosome disjunction. These data collectively support a hypothesis that Scd1 interacts with tubulins with substantial specificity to affect proper spindle formation. Finally, our genetic data suggest that the ability to affect spindle functioning is a feature specific to the Ras1-Cdc42sp, but not the Ras1-Byr2, signal transduction pathway.

*scd1*Δ is synthetically lethal with both *moe1*Δ and the tubulin mutation. The former has been shown to render microtubules abnormally stable while the latter renders them unstable. These results suggest that a dramatic increase or decrease in microtubule stability together with *scd1*Δ can impair spindle formation. By what mechanism does *scd1*Δ impair spindle formation? Interphase microtubule arrays in the Scd1 α1-tubulin double mutant appear intact at 23° (see Figure 3B), and they remain intact for at least 41 hr (our unpublished results). Therefore, unlike *moe1*Δ or the tubulin mutations tested in this study, *scd1*Δ does not appear to alter the functioning of the spindle through a global change in microtubule stability. Furthermore, we do not believe that the

loss of cell polarity *per se* is the primary cause for the abnormal spindle functioning. For example, the Scd1 tubulin double mutants after HU synchronization have an elongated cell morphology before they enter mitosis (compare cells in Figure 3B to 3D), but the formation of the spindle is markedly retarded. Similarly, those cells containing abnormal spindles and missegregated chromosomes as a result of *scd1* overexpression are also quite elongated. We note that most of the mitotic Scd1 tubulin double mutant cells contain a single microtubule dot in the nucleus. It is possible that these cells can initiate spindle nucleation but it then progresses inefficiently. Thus, we speculate that Scd1 may play a role in proper assembly of tubulin dimers into a functional spindle.

Despite the fact that Scd1 is part of a signal transduction pathway that also contains Ras1, Scd2, Cdc42, Shk1, and Moe1, Scd1 and Shk1 seem to be the only components in this complex that detectably associate with the spindle (Bauman *et al.* 1998; Sawin and Nurse 1998; Chen *et al.* 1999; Murray and Johnson 2000; S. Marcus, personal communication; our unpublished results). We speculate that Scd1 and Shk1 may be the only molecules in this complex (but not the G-proteins that regulate them) that must be anchored to the spindle, while other components interact with the spindle in a transient fashion, not detectable by our methods.

We suspect that the interaction between Scd1 and the spindle is bridged by other nuclear proteins. We have so far been unable to detect *in vitro* physical association between purified Scd1 (purified from *Sf*9 cells) and microtubules assembled from purified bovine tubulins (our unpublished results). Moreover, it is evident from the subcellular localization study that Scd1 does not seem to associate with interphase microtubules, even though Scd1 does appear in the cytosol. Because overexpression of Scd1 induced abnormal spindle formation, it is possible that Scd1 interacts with these components in a stoichiometric manner, and overexpression of Scd1 may titrate out these proteins along with their partners, both of which are important for spindle formation and chromosome separation.

The subcellular localization pattern of Scd1 is complex. We believe that this level of complexity reflects the diverse nature of the cellular events regulated by Scd1. Scd1 localizes to the cell equator, which is consistent with the fact that Scd1 genetically interacts with Byr4 and Myo2 (a type II myosin heavy chain) to affect cytokinesis (Song *et al.* 1996; Kitayama *et al.* 1997). Scd1 appears at the cell ends, which supports a hypothesis that Scd1 is among a growing list of molecules, such as Tea1 (Mata and Nurse 1997), that reside in the cell ends to maintain bipolar cell extension. Most intriguing, Scd1 also appears in the nucleus. One obvious interpretation for this is that Scd1 must enter the nucleus to interact with components necessary for spindle formation (*i.e.*, Moe1; see Chen *et al.* 1999 or unidentified spindle-binding proteins, as discussed above). Alternatively, Scd1 may interact with nuclear factors that are important for cell polarity, which has been shown to occur to the Scd1 homolog in the budding yeast, Cdc24. Cdc24 binds Far1 in the nucleus (Toenjes *et al.* 1999; Nern and Arkowitz 2000; Shimada *et al.* 2000). Upon stimulation by the mating pheromone, the Cdc24-Far1 complex exits the nucleus and, guided by Far1, binds the mating pheromone receptor. Thus the complex formation between Cdc24 and Far1 in the nucleus allows subsequent cytoskeletal reorganization to take place precisely where the mating pheromone receptor is. Future experiments are clearly needed to determine whether Scd1 behaves similarly in *S. pombe*. Meanwhile, in this study, we present evidence that Scd1, like Cdc24, may also travel between the nucleus and sites of polarization. This conclusion is deduced from the observation that Scd1 seems to translocate from the nucleus to the cell ends in the presence of Cdc42sp[T17N].

The ability to regulate organization of the actin cytoskeleton has been firmly established as a conserved feature of the Ras pathways (reviewed by Van Aelst and D'Souza-Schorey 1997). In *S. pombe*, F-actin is disorganized in *ras1* and *scd1* mutant cells (Pichová and Streiblová 1992; Snell and Nurse 1994); furthermore, the Ras1 pathway genetically interacts with Myo2. Therefore, it is highly probable that Ras1, like Ras in other systems, can regulate actin functions. Our results presented previously (Chen *et al.* 1999) and in this study indicate that the Ras1 pathway can also regulate functions of the microtubule cytoskeleton. These data raise an interesting possibility that the Ras1 signaling pathway may play a role in coordinating the functions of both actin and microtubules.

It is generally accepted that the development of cancer requires multiple mutations (Loeb 1991), and genome instability has been postulated to be a critical mechanism by which mutations favorable to cancer cells can arise. Our genetic data indicate that the Ras1-Cdc42sp pathway plays a key role in maintaining genome stability in fission yeast, and we argue that this feature of the Ras pathway is conserved in higher eukaryotes. Several recent reports indicate that oncogenic Ras can indeed induce genome instability in both mouse and human cell lines and in cancer cells (de Vries *et al.* 1993; Agapova *et al.* 1999; Saavedra *et al.* 1999), an effect that seems to be more severe in the presence of p53 mutations. To what degree are the downstream elements of Ras, which are required for maintaining genome stability in fission yeast and humans, conserved? Saavedra *et al.* (1999) have shown that oncogenic Ras induces genome instability in NIH 3T3 cells in a mitogen-activated protein (MAP) kinase (MAPK1)-dependent fashion. By contrast, we show that in fission yeast, the Byr2-MAP kinase pathway does not play a major role in maintaining genome stability; rather, it is the Cdc42sp pathway that is responsible for this function.

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