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Response of fission yeast to toxic cations involves cooperative action of the stress-activated protein kinase, Spc1/Sty1, and the Hal4 protein kinase

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ABSTRACT

Stress-activated protein kinases (SAPKs), members of a mitogen-activated protein kinase (MAPK) subfamily, are highly conserved among eukaryotes. Studies in yeasts demonstrated that SAPKs play pivotal roles in survival responses to high osmolarity, oxidative stress, and heat shock. Here we report a novel physiological role of the fission yeast Spc1 SAPK in cellular resistance to certain cations, such as Na^+, Li^+ and Ca^{2+}. Strains lacking Spc1 or its activator, Wis1 MAPKK, are hypersensitive to these cations. Spc1 positively regulates expression of *sod2*$^+$ encoding a Na^+/H^+ antiporter through Atf1 and other transcription factors. In addition, we have identified a novel Spc1-interacting protein, Hal4, which is highly homologous to the budding yeast Sat4/Hal4 protein kinase. Like its budding yeast counterpart, the fission yeast Hal4 kinase is essential for cellular resistance to Na^+, Li^+ and Ca^{2+}. The *hal4* null phenotype is complemented by overexpression of the Trk1 potassium transporter or increased K^+ in the growth medium, suggesting that Hal4 promotes K^+ uptake, which consequently increases cellular resistance to other cations. Interestingly, the Spc1-Hal4 interaction appears to be required for cellular resistance to Ca^{2+} but not Na^+ and Li^+. We propose that Spc1 SAPK and Hal4 kinase cooperatively function to protect cells from the toxic cations.

INTRODUCTION

In response to environmental fluctuations, cells modulate diverse aspects of their physiology for maximal adaptation. In eukaryotes from yeast to humans, stress-activated protein kinases (SAPKs) play key roles in cellular responses to the environment. SAPKs belong to the mitogen-activated protein kinase (MAPK) family, and their prototype, Hog1, was first identified as a component of the signaling pathway that induces cellular response to high osmolarity stress in *Saccharomyces cerevisiae* (6). In the fission yeast *Schizosaccharomyces pombe*, the Hog1 ortholog, Spc1 (also known as Sty1/Phh1), is responsive to multiple forms of environmental stress (23, 30, 50). Osmostress, oxidative stress, UV, heat shock as well as nutritional limitation induce Spc1 activation and the *spc1* null (Δ*spc1*) mutant is hypersensitive to these stress conditions, indicating the essential roles of Spc1 SAPK in cellular resistance to the stresses (11, 12, 51, 56). The same set of stresses also activate the mammalian SAPKs, p38 and JNK, which have been implicated in a variety of biological processes, including inflammation, cellular differentiation, apoptosis, cancer, and immune responses (25). Because of the evolutionary conservation of SAPKs, genetic studies in yeast have been instrumental for discovery and characterization of the SAPK regulation and function (10, 37).

Like other MAPKs, SAPKs are activated through simultaneous phosphorylation of the conserved threonine and tyrosine residues in the activation loop (61). In *S. pombe*, Spc1 is phosphorylated and activated by Wis1 MAPKK (30, 50). Wis1 is also activated through phosphorylation carried out by MAPKKKs, Wis4 and Win1 (42, 43, 49, 56), and aspartate substitutions of the MAPKKK phosphorylation sites in Wis1 (*wis1DD*) mimic phosphorylation and constitutively activate Wis1 (55). On the other hand, Spc1 is negatively regulated by dephosphorylation; tyrosine-phosphatases, Pyp1 and Pyp2 (12, 30, 50), and serine/threonine-phosphatases, PP2C (36, 52), dephosphorylate and inactivate the SAPK. A recent study indicates that Spc1 is also regulated by a molecular chaperone, Cdc37 (59).

One of the known outputs of Spc1 activation is to induce expression of genes required for cellular survival of stress conditions. Activated Spc1 translocates from the cytoplasm to the nucleus (15) and regulates gene expression through a ATF/CREB-family transcription factor, Atf1 (51, 62). Recent DNA microarray experiments demonstrated that the Spc1-Atf1 pathway is required for the majority of the Core Environmental Stress Response (CESR) genes, ~140 genes that are induced under multiple stress conditions (9). The CESR genes comprise those involved in a variety of cellular processes, including carbohydrate and lipid metabolism, DNA repair, protein folding/degradation, antioxidants, and transcription, illustrating that the cellular stress response encompasses modulation of diverse cellular processes. In addition to Atf1, Spc1 SAPK also interacts and phosphorylates two protein kinases, Cmk2 and Srk1 (4, 44, 57), although the contribution of these kinases to cellular stress resistance is less obvious.

Here we report a novel physiological role of Spc1 SAPK; Spc1 activity is essential for cellular resistance to Na^+, Li^+ and Ca^{2+}, cations of which concentrations are normally maintained relatively low in the cytoplasm. Spc1 positively regulates expression of the *sod2*$^+$ gene encoding a plasma membrane Na^+/H^+ antiporter, which exports Na^+ and Li^+ (22). In addition, through a yeast two-hybrid screen, we have isolated a novel protein kinase that physically interacts with Spc1. This kinase has been named Hal4, because of its high similarity to the budding yeast Sat4/Hal4 kinase, which is known to play a role in cellular salt tolerance (33). Like Δ*spc1*, the *S. pombe* Δ*hal4* mutant shows hypersensitivity to Na^+, Li^+ and Ca^{2+}. The multi-copy suppressor screen of the Δ*hal4* cation sensitivity has isolated the *trk1*$^+$ potassium transporter gene, and potassium added to the growth medium complements the Δ*hal4* phenotypes. These results suggest that Hal4 kinase promotes K^+ uptake, which increases the cellular resistance to other toxic cations. We propose that Spc1 SAPK and Hal4 kinase act cooperatively for cellular cation homeostasis.

MATERIALS AND METHODS

Yeast strains and cultures.

S. pombe strains used in this study are listed in Table 1. Growth media and basic techniques for *S. pombe* have been described previously (2, 32). For construction of the *spc1KM* strain, Lys-49 of the ATP-binding site in Spc1 was mutated to Met by the QuikChange XL site-directed mutagenesis kit (Stratagene) in the *spc1*$^{+}$ DNA fragment, which was used to transform the *spc1::ura4*$^{+}$ strain (CA1366). Ura^{-} transformants were selected by 5-fluorootic acid (5FOA)-containing medium and replacement of the *spc1::ura4* locus with *spc1KM* was confirmed by Southern hybridization. Expression of the Spc1KM protein was also confirmed by anti-Spc1 immunoblotting.

S. pombe cells were grown in yeast extract medium YES and synthetic minimal medium EMM2. NaCl, KCl, LiCl and $CaCl_2$ were added in YES medium as indicated. Salt tolerance was determined by drop test using saturated culture diluted to OD_{600} 1.0 and subsequent 10 folds serial dilutions. SD minimal medium used for growth test was previously described (48).

Purification and detection of the Spc1HA6H protein

KS1376 (*spc1:HA6H*) cells growing at 30^{o}C in YES medium were stressed with 0.6 M or 0.1 M KCl and 0.1 M NaCl, and harvested along the time course by rapid filtration (54). The Spc1-HA6H protein was purified under the denaturing condition and analyzed by immunoblotting with anti-phospho-p38 antibodies (54).

Multicopy suppressor screens.

To isolate multicopy suppressors that complement the Li-sensitivity of the Δ*spc1* mutant, strain KS1366 (Δ*spc1*) was transformed with a *S. pombe* genomic library in the pAL-KS vector (58) and plated onto EMM2 agar plates supplemented with uracil, adenine and histidine. After incubation at 30°C for 4 days, transformants were replica-plated onto YES agar plates containing

4 mM LiCl. Colonies that showed plasmid-dependent resistance to LiCl were isolated and plasmids recovered from these colonies were analyzed by DNA sequencing. The multicopy suppressor screen with the Δ*hal4* strain was performed similarly, except that a lower concentration of LiCl (2 mM) was used for selection.

Isolation of the *hal4*$^+$ gene by yeast two-hybrid screens.

The *spc1TA* mutant gene, in which Thr-171 is substituted with Ala, was constructed by the QuikChange XL site-directed mutagenesis kit and used as bait in a yeast two-hybrid screen to isolate Spc1 interacting proteins from the *S. pombe* two-hybrid cDNA library in the pGADGH vector (18). Yeast two-hybrid assay was carried out in *S. cerevisiae* HF7c strain (14) by His$^+$ selection and the β-galactosidase assay. From ~1 X 10^7 transformants screened, three different plasmids containing the *hal4*$^+$ cDNA were isolated.

Plasmids construction.

Sequences of the PCR primers used in this study are available upon request. All PCR-amplified fragments were confirmed by DNA sequencing.

The *hal4*$^+$ open reading frame (ORF) was amplified by PCR using *S. pombe* genomic DNA as template and cloned into the pREP1 (29) and pREP1-KZ vectors (53). The QuikChange XL site-directed mutagenesis kit was used to introduce a point mutation into the *hal4*$^+$ gene to construct the *hal4KM* allele. An internal deletion mutant of *hal4*, Δ(181-230), was constructed by a PCR-based mutagenesis method previously described (24). For the yeast two-hybrid assays to characterize interaction between Hal4 and Spc1, *hal4* coding sequences for full length (1-636), residues 181-350, 231-636, 181-300, 231-350, and 181-250 were amplified by PCR with *S. pombe* genomic DNA as template and cloned into the pGADGH vector. The wild-type *spc1*$^+$ gene was used as bait. To express the full-length and the C-terminally truncated Trk1, the entire ORF (residues 1-841) as well as the sequence encoding residues 1-803 were amplified by PCR and cloned into the *S. pombe* expression vector pNMT1 (Invitrogen).

Construction of *hal4*:*myc* strain.

For constructing strains in which the chromosomal *hal4*$^+$ gene is tagged with the sequences encoding twelve copies of the myc epitope, a *Not*I site was introduced by PCR at the 3' end of the *hal4*$^+$ ORF. The resultant PCR fragment of the 3'-terminal, 648-bp *hal4*$^+$ ORF was used to construct the pBluescript-ΔNHal4-Myc plasmid with the *ura4*$^+$ marker gene. The plasmid was linearized at a *Xba*I site within the *hal4* ORF and used to transform a wild-type (PR109) strain, and the integration of the plasmid at the *hal4*$^+$ locus were confirmed by Southern blotting analysis. The cation resistance of the resultant *hal4:myc* strain was comparable to that of wild-type cells, indicating that the *hal4:myc* fusion gene is functional.

Immunoprecipitation.

hal4:myc (CA1998), *hal4:myc* Δ*spc1* (CA2021) and *cmk2:myc* (KS2012) cells, grown to mid-log phase at 30°C in YES medium, were harvested before and after stress treatments by rapid filtration (54). Cells were lysed in lysis buffer (50 mM HEPES pH7.4, 2 mM EDTA, 2 mM $MgCl_2$, 10% glycerol, 1% NP-40, 1 mM PMSF, 50 mM NaF and 0.1 mM Na_3VO_4). Anti-Spc1 rabbit polyclonal antibodies conjugated to protein A-Sepharose (Pharmacia Biotech) were used to precipitate Spc1 protein from the lysate, which was followed by immunoblotting with anti-Spc1 and mouse monoclonal anti-myc antibodies (9E10, Boehringer Mannheim).

Gene disruption.

For *hal4* gene disruption, the 0.5-kb genomic sequences immediately upstream and downstream of the *hal4*$^+$ ORF were amplified by PCR with pairs of primers, sp27-f and sp27-fcT7 for the upstream sequence, and T3sp27-b and sp27-bc for the downstream sequence. Subsequently, these fragments were ligated to the 5' and 3' ends of the 1.8-kb *ura4*$^+$ marker gene fragment through another PCR reaction. The resultant PCR product, a *ura4*$^+$ fragment flanked by the genomic sequences adjacent to *hal4*$^+$ were used for transformation. Stable Ura$^+$ transformants

were isolated and the disruption of the *hal4* locus was confirmed by Southern hybridization experiments. Similar procedures were used in the *trk1* and *trk2* gene disruptions. The PCR primers used were U5trk1 and U3trk1 to amplify the sequence upstream of *trk1*$^+$ and D5trk1 and D3trk1 to amplify the sequence downstream of *trk1*$^+$. The *trk2*$^+$ upstream sequence was amplified with primers U5trk2 and U3trk2, and the *trk2*$^+$ downstream sequence was amplified with D5trk2 and D3trk2 primers. Instead of *ura4*$^+$, *kanMX6* (5) was used as marker gene and transformants were selected on YES agar plates containing 0.1 mg/ml G418.

RESULTS

Inactivation of the Spc1 MAPK cascade brings about hypersensitivity to toxic cations. Mutants lacking functional Spc1 SAPK or Wis1 MAPKK are hypersensitive to high osmolarity stress, indicating the Spc1 cascade is essential for cellular responses to high osmolarity stress (30, 50, 52). During further characterization of the osmostress response of *S. pombe* cells using various salts as osmolyte, we noticed that the *spc1* null (Δ*spc1*) mutant is hypersensitive to even low concentrations of NaCl. While wild-type cells grow in the presence of 0.5 M NaCl (data not shown; (22)), even 0.1 M NaCl in the growth medium caused an apparent growth defect in the Δ*spc1* mutant (Fig. 1A). The same concentration of KCl did not inhibit the growth of Δ*spc1* cells, suggesting that the observed growth inhibition is due to Na^+, rather than Cl^- or osmolarity. Because the Δ*wis1* MAPKK mutant also exhibited very similar Na^+-sensitivity (Fig. 1A), the activity of Spc1 MAPK appears to be required for cellular resistance to Na^+. The requirement of the Spc1 kinase activity for the cation resistance was further confirmed by the *spc1KM* strain, which expresses a catalytically inactive Spc1 SAPK with a point-mutation in its ATP-binding site; *spc1KM* mutant cells are sensitive to high osmolarity of 1 M KCl as well as to low concentrations of Na^+ (Fig. 1B). We therefore examined whether cells induce activation of Spc1 SAPK in response to Na^+ stress. In contrast to high osmolarity stress by 0.6 M KCl, addition of 0.1 M NaCl to the growth medium brought about only weak, transient Spc1 activation comparable to that by 0.1 M KCl (Fig. 1C), probably due to mild osmostress. Thus, although Spc1 activity is essential for cellular survival of Na^+ stress, Na^+ is not an activating stimulus for the SAPK.

Survey of other metal ions demonstrated that the Δ*spc1* mutant is also hypersensitive to Li^+ and Ca^{2+} (Fig. 1A). To further confirm that the Spc1 cascade has an active role in protecting cells from these cations, we examined the cation sensitivity of a strain expressing the mutant Wis1DD MAPKK. Wis1DD has aspartate substitutions at the MAPKKK phosphorylation sites and constitutively activate Spc1 SAPK (55). As shown in Fig. 1D, the *wis1DD* allele conferred

higher resistance to the toxic cations. We also observed elevated cation resistance with a strain lacking the Pyp1 tyrosine phosphatase (data not shown), a negative regulator of Spc1 SAPK (30, 50). These results strongly suggest that the Spc1 cascade positively regulates cellular resistance to the cations.

The Atf1 transcription factor contributes to cellular cation resistance.

To understand how Spc1 SAPK regulates cellular responses to the toxic cations, we next examined whether the known Spc1 substrates are required for the cation resistance. The Atf1 transcription factor is phosphorylated by Spc1 upon stress and regulates a number of stress-resistance genes (51, 62). The Δ*atf1* strain was not significantly sensitive to 0.1 M NaCl, although it shows moderate Li^+-sensitivity (Fig. 1A). On the other hand, Atf1 is clearly important for the increased Na^+- and Li^+-resistance of *wis1DD* cells, because the *wis1DD* Δ*atf1* double mutant is no more resistant to these cations than wild-type cells (Fig. 1D). As previously reported (39), Δ*atf1* cells are very sensitive to Ca^{2+}, a phenotype severer than that of the Δ*spc1* strain (Fig. 1A). Thus, the cation sensitive phenotypes of Δ*spc1* and Δ*atf1* are not identical, implying that, in addition to Atf1, Spc1 may function also independently of Atf1 in cellular responses to the toxic cations.

Two protein kinases, Cmk2 and Srk1, have recently been reported as downstream targets of Spc1 SAPK (4, 44, 57). The Δ*cmk2* Δ*srk1* double mutant (Fig. 1A) as well as the individual single mutants (data not shown) did not show altered sensitivity to Na^+, Li^+ and Ca^{2+}, indicating that these Spc1 substrates are not involved in the cellular cation resistance.

Spc1 SAPK regulates expression *sod2*$^+$, a Na^+/H^+ antiporter gene.

Aiming to discover how Spc1 affects the cellular resistance to the toxic cations, we performed two screens, a multicopy suppressor screen of the Δ*spc1* mutant and a yeast two-hybrid screen with Spc1 as bait. In the former screen, we screened a *S. pombe* genomic library for plasmid clones that rescue the Li^+-sensitive phenotype of the Δ*spc1* mutant (Materials and Methods).

Other than the plasmids carrying *spc1*$^+$ itself, this screen repeatedly isolated plasmids that contain the *sod2*$^+$ gene encoding a Na^+/H^+ antiporter in the plasma membrane (22) (Fig. 2A). Sod2 exports Na^+ and Li^+, and increased expression of Sod2 confers cellular resistance to these cations (22). Indeed, the multi-copy *sod2*$^+$ plasmid complemented both Li^+- and Na^+-sensitivities of Δ*spc1* cells (Fig. 2A) but not their Ca^{2+}-sensitive phenotype (data not shown). In order to test the possibility that *sod2*$^+$ expression is regulated by Spc1 SAPK, the *sod2*$^+$ mRNA level was quantified by Northern blotting in wild-type and Δ*spc1* strains. The *sod2*$^+$ mRNA level in the Δ*spc1* mutant was reduced to ~50% of that in wild-type cells (Fig. 2B), indicating that Spc1 SAPK positively regulates *sod2*$^+$ expression. Consistent with this idea, *sod2*$^+$ expression was increased by ~30% in the *wis1DD* mutant, which expresses activated Wis1 MAPKK. The *sod2*$^+$ mRNA level in the Δ*atf1* strain was ~75% of that in wild-type cells, implying that Atf1 partly contributes to *sod2*$^+$ transcription. These results suggest that Spc1 SAPK regulates *sod2*$^+$ expression through Atf1 and other transcription factors and that the reduced *sod2*$^+$ level in the Δ*spc1* mutant contributes to its Na^+- and Li^+- sensitive phenotypes.

Isolation of Hal4 protein kinase as a novel Spc1-interacting protein.

In the yeast two-hybrid screen for proteins interacting with Spc1, the cDNA of Cmk2, a known substrate of Spc1 (4, 44), as well as plasmids carrying the open reading frame (ORF) SPAC29A4.16 were isolated. SPAC29A4.16 encodes a putative serine-threonine protein kinase most homologous to the *S. cerevisiae* Sat4/Hal4 kinase (33); therefore, we termed this *S. pombe* gene *hal4*$^+$. The *hal4*$^+$ gene encodes a 636-amino acid, 69-kDa protein with a C-terminal kinase catalytic domain. The amino acid sequence similarity between the *S. pombe* Hal4 and *S. cerevisiae* Sat4 proteins is mostly limited to their catalytic domains with 67% identity (Fig. 3A, shaded). Published genome data of other fungi, including *Candida*, *Aspergillus* and *Neurospora*, also contain protein kinases with high similarities in their catalytic domains (data not shown). On the other hand, little similarity was detected in the N-terminal, non-catalytic domains. In addition

to Sat4, *S. cerevisiae* has another homologous protein kinase, Hal5 (33), but no apparent paralog was found in the *S. pombe* genome.

The shortest *hal4* cDNA clone isolated in the two-hybrid screen lacked the sequence for the N-terminal 180 residues (181-636 in Fig. 3B). We confirmed that the full length (1-636) Hal4 also interacts with Spc1 in the two-hybrid assay. Further truncation analyses of the *hal4* sequence in the same assay demonstrated that residues 180-230 in the non-catalytic domain are critical for interaction with Spc1 (Fig. 3B). This region does not share any apparent similarity with the previously reported MAPK-docking sequences (21, 47).

Interaction between Spc1 SAPK and Hal4 kinase was also confirmed biochemically. We inserted the myc epitope sequence at the 3' end of the chromosomal *hal4*$^+$ ORF, so that the Hal4-myc fusion protein is expressed from the *hal4* locus. Unlike the Δ*hal4* strain (see below), the resultant *hal4:myc* strain shows cation resistance comparable to that of *hal4*$^+$ cells (data not shown), indicating that the Hal4-myc fusion protein is functional. Immunoprecipitation of Spc1 from the *hal4:myc* cell lysate co-purified Hal4-myc, while similar procedure using Δ*spc1 hal4:myc* cells resulted in little precipitation of Hal4-myc (Fig. 4). Stress conditions that activate Spc1 SAPK, such as osmostress, did not affect the Hal4-Spc1 interaction. Although co-precipitation of Hal4 with Spc1 was not as efficient as another Spc1-interacting kinase, Cmk2, under the experimental conditions tested (Fig. 4), these biochemical data corroborated the *in vivo* interaction between Hal4 and Spc1 SAPK.

Hal4 kinase does not appear to be a regulator of Spc1 activation. Quantification of Spc1 activation by in *hal4*$^+$ and Δ*hal4* strains showed comparable activation of Spc1 in response to the known Spc1-activating stresses (data not shown).

Hal4 protein kinase is required for cellular resistance to the toxic cations.

The *S. cerevisiae SAT4/HAL4* and its paralog, *HAL5*, were isolated for their ability to confer halotolerance to wild-type cells upon overexpression, and the null mutants of these genes exhibit hypersensitivity to Na^+, Li^+ and Ca^{2+} (33). To test whether *S. pombe* Hal4 is also required for

cellular resistance to these cations, we constructed the *hal4* null (Δ*hal4*) mutant by gene disruption. Haploid Δ*hal4* cells were viable and showed no apparent growth defect in both rich and minimal growth media (data not shown). However, their growth was impaired in the presence of Na^+, Li^+ and Ca^{2+}, and these Δ*hal4* phenotypes were even severer than those of the Δ*spc1* mutant (Fig. 5A). On the other hand, in contrast to Δ*spc1*, the Δ*hal4* mutant is not sensitive to high osmolarity stress of 1 M KCl. These results indicate that Hal4 protein kinase plays an important role in cellular resistance to Na^+, Li^+ and Ca^{2+}. We noticed that the Δ*spc1* Δ*hal4* double mutant is more sensitive to those cations than the individual single mutants are (Fig. 5A). This observation suggests that the functional relationship between Spc1 and Hal4 in the cellular cation response is not simply through a linear pathway; both Spc1 activation as well as Hal4 function are required for proper cellular responses to Na^+, Li^+ and Ca^{2+}. Consistently, overexpression of Hal4 did not complement the Δ*spc1* cation sensitivity, and overexpression of Spc1 did not rescue the Δ*hal4* phenotype (data not shown).

The importance of the protein kinase activity of Hal4 for its function was confirmed by constructing a catalytically inactive Hal4 mutant. Lys-385 within the ATP-binding domain of Hal4 was replaced with methionine by site-directed mutagenesis. Expression of this Hal4KM protein as a fusion with a GST (glutathione-*S*-transferase)-tag did not rescue the cation sensitivity of the Δ*hal4* strain, while wild-type GST-Hal4 complemented the Δ*hal4* defect (Fig. 5B). Thus, Hal4 functions as a protein kinase to protect cells from the cation stress.

To test whether the interaction with Spc1 is important for the Hal4 function, the mutant Hal4 lacking the Spc1-binding domain (Δ181-230, Fig. 3B) was expressed in the Δ*hal4* mutant. Like the wild-type *hal4*$^+$ gene, the mutant *hal4*Δ*(181-230)* gene complemented the Na^+ and Li^+ sensitivity of Δ*hal4* cells, indicating that the mutant Hal4 kinase is active even without the Spc1-binding domain (Fig. 5C). Strikingly, however, the *hal4*Δ*(181-230)* expressing cells were found to be sensitive to Ca^{2+}, indicating that this mutant kinase cannot replace the Hal4 function in cellular resistance to Ca^{2+}. These results imply that the Spc1-Hal4 interaction is required for cell viability in the presence of Ca^{2+} but dispensable for resistance to Na^+ and Li^+.

Hal4 protein kinase is involved in cellular potassium homeostasis.

Aiming to obtain a clue for the physiological function of Hal4 kinase, we next performed a multicopy suppressor screen to isolate genes whose overexpression rescues the cation sensitivity of Δ*hal4* cells (Materials and Methods). Screening of 6 x 10^4 genomic library transformants identified 76 plasmids containing *hal4*$^+$ itself and one plasmid carrying the *trk1* gene encoding a major potassium transporter (7, 27). The isolated *trk1* clone lacked the 3'-end of the ORF for the C-terminal 38 amino acid residues, but complemented the cation sensitive phenotypes of the Δ*hal4* strain to the levels comparable to the complementation by the *hal4*$^+$ plasmid (Fig. 6A). In order to test the effect of the C-terminal 38-residue truncation, the full-length and the C-terminally truncated *trk1* ORFs are amplified by PCR and cloned into an expression vector. As shown in Fig. 6B, expression of the full-length Trk1 only weakly complemented the *hal4* phenotypes, comparing to the strong suppression by the Trk1 lacking its C-terminal 38 residues. Trk1 has twelve membrane-spanning sequences with its N- and C-termini are predicted to be in the cytoplasm (7, 27), and these results may suggest a role of the C-terminal cytoplasmic domain in the regulation of Trk1. On the other hand, the cation sensitivity of Δ*spc1* cells was not significantly rescued by plasmids expressing the full-length (data not shown) or C-terminally truncated Trk1 (Fig. 6C), implying that Hal4 and Spc1 contribute to the toxic cation resistance through a distinct mechanism.

The observation above suggests that the Hal4 function may be related to K^+ transport. Interestingly, studies in budding yeast demonstrated that cellular sensitivity to the toxic cations is significantly affected by cellular potassium homeostasis. The plasma membrane H^+-ATPase generates electrical membrane potential, negative inside, which is counteracted by K^+ uptake (46). The plasma membrane is hyperpolarized in mutants defective in K^+ transport, leading to higher rates of other cation uptake across the electrical gradient (28); therefore, those mutants are sensitive to even low concentrations of Na^+, Li^+ and Ca^{2+}. Based on genetic analyses, it was proposed that *S. cerevisiae SAT4* positively regulates K^+ uptake through two major potassium

transporters Trk1 and Trk2 (33). Therefore, we further examined whether *S. pombe* Hal4 kinase is involved in cellular potassium uptake.

We found that the Δ*hal4* mutant grows poorly in synthetic SD medium, which contains only 7 mM of K^+ (Fig. 7A). This growth defect was significantly rescued by supplementing the growth medium with 0.2 M KCl, an observation consistent with the idea that Hal4 plays a role in K^+ uptake. In contrast, Δ*spc1* cells exhibited no apparent growth defect in SD medium (data not shown), suggesting that Spc1 SAPK is not required for K^+ uptake. Furthermore, addition of K^+ to the growth medium was found to rescue the cation sensitive phenotypes of Δ*hal4* cells. In the presence of 50 mM KCl, the resistance of the Δ*hal4* mutant to the toxic cations was comparable to that of wild-type cells (Fig. 7B). The Δ*trk1* mutant is also hypersensitive to Na^+ (7) and Li^+ (Fig. 7B), but this phenotype was rescued by additional K^+ in the growth medium. Together, these results strongly suggest that, also in *S. pombe*, Hal4 kinase positively regulates K^+ uptake.

Like in *S. cerevisiae*, the *S. pombe* genome contains two genes, *trk1*$^+$ and *trk2*$^+$, encoding evolutionarily conserved TRK-family K^+ transporters (7, 27). To test whether the *S. pombe* Hal4 kinase regulates K^+ uptake through the TRK transporters as proposed for *S. cerevisiae* Sat4, we examined genetic interactions between the *hal4*$^+$ and *trk*$^+$ genes. Genetic crosses between *hal4*::*ura4*$^+$ (Δ*hal4*) and *trk1*::*kanR* (Δ*trk1*) produced no viable progeny of Δ*hal4* Δ*trk1*; spores expected to carry both mutations often failed to germinate or ceased to grow immediately after germination (data not shown). Therefore, by crossing the Δ*trk1* mutant with a Δ*hal4* strain transformed by a *hal4*$^+$ plasmid, viable Δ*hal4* Δ*trk1* double mutant cells carrying the *hal4*$^+$ plasmid was isolated. Loss of the *hal4*$^+$ plasmid was observed only when these cells were grown in rich medium supplemented by high concentrations of K^+, such as 1 M KCl (data not shown), indicating the suppression of the Δ*hal4* Δ*trk1* lethality by high levels of external K^+. In contrast, the Δ*trk1* Δ*trk2* double mutant was found to be viable even in regular growth media, as previously reported (8), although its growth was significantly slower than wild-type cells unless the growth medium was supplemented by 0.2 M KCl (data not shown). These observations do not negate Trk regulation by Hal4, but the severer growth defect of the Δ*hal4* Δ*trk1* double

mutant than the Δ*trk1* Δ*trk2* mutant indicates that *S. pombe* Hal4 may also have a function independent of Trk1 and Trk2.

DISCUSSION

Alkali cations, such as K^+, Na^+, and Ca^{2+} are vital elements that play multiple roles in all living cells, whereas these cations must be maintained within a limited concentration range to avoid toxicity. For example, the concentration of Na^+ is kept relatively low in many cell types, while K^+ is found at much higher concentrations, since some metabolic enzymes are known to be sensitive to Na^+ as well as Li^+ ions (1, 13, 34, 35). Although NaCl was used as osmolyte to isolate and characterize mutants of the osmosensing Hog1 SAPK in *S. cerevisiae*, we noticed that the homologous SAPK in *S. pombe*, Spc1, is required for cellular resistance to Na^+, independently of its role in the osmostress response. Cells lacking functional Spc1 are hypersensitive to Na^+, Li^+ and Ca^{2+}, while strains with increased Spc1 activity exhibits enhanced resistance to these cations. The crucial roles of SAPK in cellular responses to osmostress, oxidative stress and heat shock have been well studied; however, as far as we are aware of, this is the first to clearly demonstrate the requirement of SAPK for cellular salinity resistance.

One of the major functions of Spc1 SAPK is to induce expression of stress resistance genes through the Atf1 transcription factor (51, 62). Indeed, the Δ*atf1* mutant is hypersensitive to Ca^{2+} and also shows mild sensitivity to Na^+ and Li^+. In addition, the hyper-resistance to Na^+ and Li^+ in the *wis1DD* strain was compromised in the Δ*atf1* background. These data suggest that transcriptional regulation by Atf1 partly contributes to cellular resistance to the cations. Previous DNA microarray studies demonstrated that the Spc1-Atf1 pathway regulates the majority of the Core Environmental Stress Response (CESR) genes inducible in response to multiple forms of stress (9). However, from their sequence information, none of the CESR genes seem to have apparent involvement in cellular resistance to the cations. In this study, we isolated *sod2*$^+$ as a

multi-copy suppressor of the Li^+ - and Na^+-sensitive phenotypes of the Δ*spc1* mutant. *sod2*$^+$ encodes a Na^+/H^+ antiporter that exports Na^+ and Li^+ at the plasma membrane, and its expression level is known to correlate with cellular resistance to these cations (22). We found that the *sod2*$^+$ mRNA level is reduced in the Δ*spc1* mutant and increased in the *wis1DD* strain, observations consistent with the idea that *sod2*$^+$ is a novel transcriptional target of the Spc1 pathway. *sod2*$^+$ expression showed only a minor reduction in the Δ*atf1* mutant, and therefore, Atf1 may not be the only transcription factor that mediates *sod2*$^+$ regulation by Spc1. It was previously reported that the Spc1-Atf1 pathway is responsible for salt-induced expression of *cta3*$^+$ (17, 38), which encodes a cation-efflux ATPase homologous to the budding yeast Ena1 (16). Although Ena1 is essential for Na^+/Li^+-resistance of budding yeast cells (19), the Δ*cta3* mutant is not sensitive to Na^+, Li^+ and Ca^{2+} (17, 38; our unpublished results), and the physiological role of Cta3 is unclear.

The involvement of Spc1 SAPK in the cellular response to the cations has been further corroborated by the discovery of its physical interaction with Hal4 kinase. Its budding yeast orthologs, Sat4/Hal4 and Hal5, are essential for cellular resistance to the toxic cations (33), and consistently, we have demonstrated that the *S. pombe* Δ*hal4* mutant is hypersensitive to Na^+, Li^+ and Ca^{2+}. How does Hal4 kinase modulate cellular cation resistance? Studies in budding yeast suggest that Sat4/Hal4 and Hal5 kinases positively regulate cellular potassium uptake, which reduces influx of the toxic cations as follows. The plasma membrane H^+-ATPase generates electrochemical proton gradient across the membrane, negative inside, which drives the secondary transport of nutrients as well as influx of cations (45). This membrane potential is counteracted by cellular K^+ uptake, mainly through the Trk1 and Trk2 transporters; deletion of the *TRK1* and *TRK2* genes results in the hyperpolarized plasma membrane potential and increased uptake of cations (28), leading to cellular hypersensitivity to the toxic cations (33). Similar phenotypes were observed with the budding yeast *sat4 hal5* mutant and, from epistasis analyses, it was proposed that Sat4/Hal4 and Hal5 kinases positively regulate K^+ uptake through the Trk transporters (33). Our data also implicate the *S. pombe* Hal4 kinase in cellular K^+ uptake. First, the multi-copy suppressor screen of the Li^+-sensitivity in the Δ*hal4* mutant isolated the

trk1$^+$ potassium transporter gene. Second, the Δ*hal4* mutant shows a growth defect in K^+-limited growth media. Third, like Δ*trk1*, Δ*hal4* cells show cation-sensitive phenotypes that are complemented by additional potassium in the growth medium. Lastly, we found that Δ*hal4* cells are hypersensitive also to the cationic antibiotics, such as hygromycin B and G418, as well as tetramethylammonium (our unpublished results), a phenotype indicative of the hyperpolarized plasma membrane (3, 33, 40). Together, these data strongly suggest that the *S. pombe* Hal4 kinase positively regulates cellular K^+ uptake, and that the loss of Hal4 activity leads to the cation sensitive phenotype probably due to hyperpolarization of the plasma membrane. As proposed in budding yeast, Hal4 may directly or indirectly regulate the Trk transporters. This regulation does not seem to be transcriptional, because the *trk1*$^+$ mRNA level is not reduced in the Δ*hal4* mutant (our unpublished results). Interestingly, expression of the truncated Trk1 without C-terminal 38 residues, but not the full-length protein, can complement the *hal4* defects. An intriguing possibility is that this cytoplasmic tail of the Trk1 transporter may be a negative regulatory domain and that the Hal4 function may be to relieve this negative regulation of Trk1. On the other hand, we found that the Δ*hal4* Δ*trk1* double mutant is lethal, while the Δ*trk1* Δ*trk2* cells are viable. Thus, in contrast to the budding yeast Sat4/Hal4, Hal4 kinase in *S. pombe* appears to have an additional function independent of the Trk transporters.

Although Spc1 physically interacts with Hal4 and the Δ*spc1* mutant shows cation sensitive phenotypes similar to those of Δ*hal4*, Spc1 SAPK does not appear to be important in cellular K^+ uptake; *Δspc1* cells exhibit no growth defect under K^+-limited conditions. In addition, overexpression of the *trk1*$^+$ gene does not complement the *Δspc1* phenotypes. Therefore, Spc1 might modulate cellular resistance to the toxic cations independently of the Hal4 function in K^+ homeostasis. Alternatively, the Spc1-Hal4 interaction might become important for cellular K^+ homeostasis only under certain conditions. We found that cells expressing the mutant Hal4 lacking the Spc1-binding domain are hypersensitive to Ca^{2+}, but not to Na^+ and Li^+, implying that cellular responses to high concentrations of Ca^{2+} may require additional regulation of Hal4 by Spc1. The molecular mechanisms in which Spc1 regulates the Hal4 function need further

investigation. The *in vivo* phosphorylation state of Hal4 did not appear to be significantly affected by the Δ*spc1* mutation, and Spc1 failed to phosphorylate Hal4 *in vitro* (our unpublished results), suggesting that Spc1 may not regulate Hal4 through phosphorylation. Spc1 also does not seem to affect the localization of the Hal4 protein, because cytoplasmic localization of Hal4 is not altered by osmostress that induces nuclear translocation of Spc1 SAPK (our unpublished results). However, the physical interaction may provide necessary coordination between the Spc1 and Hal4 functions for maximum resistance to the toxic cations. It is also possible that the Spc1–Hal4 interaction might play a role during the environmental stress responses mediated by Spc1 SAPK. In bacteria, K^+ uptake is part of the cellular response to high osmolarity stress (26, 60). Thus, it is conceivable that, in addition to transcriptional regulation, Spc1 SAPK may modulate cellular cation homeostasis through interaction with Hal4 kinase under environmental stresses.

In this study, we have identified a novel physiological role of Spc1 SAPK in cellular resistance to the toxic cations. Spc1 regulates expression of a Na^+/H^+ antiporter and also physically interacts with Hal4 kinase that regulates cellular K^+ uptake. During preparation of this manuscript, it was reported that the budding yeast Hog1 SAPK regulates the Na^+/H^+ antiporter in the plasma membrane through phosphorylation (41), indicating that budding yeast also utilizes SAPK in the cellular cation response. Considering the large evolutional distance between fission and budding yeasts (20), involvement of SAPK in cellular cation homeostasis might be conserved also in other eukaryotes. Further genetic and biochemical studies in these yeast systems will be very useful to characterize this novel SAPK function at a molecular level.

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FIGURE LEGENDS

FIG. 1. The Spc1 MAPK cascade is required for cellular resistance to the toxic cations. (A) Wild-type (KS1598), Δ*spc1* (KS1366), Δ*wis1* (JM544), Δ*atf1* (KS1497) and Δ*cmk2* Δ*srk1* (CA2685) strains were grown in liquid YES medium, and serial dilutions were spotted on YES agar plates containing 0.1 M KCl, 0.1 M NaCl, 4 mM LiCl, and 80 mM $CaCl_2$. (B) The *spc1KM* strain (CA3081), which expresses a catalytically inactive Spc1 mutant kinase, was grown in YES medium and serial dilutions were spotted on YES plates with 1 M KCl for osmostress, 0.1 M KCl and 0.1 M NaCl. (C) The *spc1:HA6H* strain (KS1376) grown to the mid-log phase at 30^{o}C in YES medium was treated with 0.1 M KCl and 0.1 M NaCl, and aliquots were harvested at the indicated times. The Spc1HA6H protein was purified under the denaturing condition and analyzed by immunoblotting with anti-phospho-p38 antibodies, which specifically recognize the phosphorylated, active form of Spc1 MAPK. (D) The cation sensitivity of the *wis1DD* (KS2081) and *wis1DD* Δ*atf1* (CA363) strains were tested by the spot test on YES plates with 0.1 M KCl, 0.1 M NaCl and 4 mM LiCl. These strains express the Wis1DD mutant MAPKK, in which the MAPKKK phosphorylation sites, Ser-469 and Thr-473, were substituted with aspartic acid for constitutive activation.

FIG. 2. Spc1 positively regulates expression of *sod2*$^{+}$ encoding a Na^{+}/H^{+} antiporter. (A) *sod2*$^{+}$ was isolated as a multi-copy suppressor of the Δ*spc1* Li^{+}-sensitivity. The Δ*spc1* strain (CA1366) carrying an empty vector and a *sod2*$^{+}$ plasmid isolated from the multi-copy suppressor screen was grown in selective EMM medium and serial dilutions were spotted on YES plates with 4 mM LiCl and 0.1 M NaCl. (B) *sod2*$^{+}$ Northern blotting of wild-type (CA1598), Δ*spc1* (CA1366), Δ*atf1* (KS1497) and *wis1DD* (KS2081) strains. Poly-A^{+} RNA was hybridized with ^{32}P-labeled *sod2*$^{+}$ probe. Signal levels quantified and normalized by control hybridization with the *leu1*$^{+}$ probe are indicated below each lane.

FIG. 3. *S. pombe* Hal4 is highly homologous to the *S. cerevisiae* Sat4/Hal4 protein kinase. (A) Amino acid sequence alignment between the kinase domains of *S. pombe* Hal4 and *S. cerevisiae* Sat4/Hal4. Identical residues are shaded. (B) The Spc1-binding domain within *S. pombe* Hal4 was determined by testing the interaction of truncated Hal4 constructs with Spc1 in the yeast two-hybrid assay. Residues from 181 to 230 (represented by a hatched box) are required for binding to Spc1.

FIG. 4. Hal4 kinase physically associates with Spc1 SAPK. The *hal4:myc* (CA1998) and *hal4:myc* Δ*spc1* (CA2021) strains, which express Hal4 fused to the myc epitope, were grown to the mid-log phase at 30°C in YES medium. Aliquots of cells were harvested before (-) or after 10-min high osmolarity stress by 0.6 M KCl (Os), and immunoprecipitates by anti-Spc1 antibodies (IP) were analyzed by Western blotting with anti-myc and anti-Spc1 antibodies. The Hal4 protein levels in the crude lysate used in this experiment were also examined by anti-myc immunoblotting (bottom panel). The *cmk2:myc* strain (KS2012) expressing the Cmk2-myc fusion protein was used as a positive control.

FIG. 5. Hal4 kinase is required for cellular resistance to Na^+, Li^+ and Ca^{2+}. (A) The growth phenotypes of wild-type (KS1598), Δ*spc1* (KS1366), Δ*hal4* (CA1787) and Δ*spc1* Δ*hal4* (CA1992) strains were examined as described in Fig. 1, except lower concentrations of LiCl (2 mM) and $CaCl_2$ (20 mM) were added to YES agar plates. Unlike Δ*spc1*, the Δ*hal4* mutant is not sensitive to high osmolarity of 1 M KCl. (B) The protein kinase activity of Hal4 is required for the toxic cation resistance. The *Δhal4* (CA1787) strain expressing GST (pGST) alone, wild-type (pGST-*hal4*) or kinase-inactive (pGST-*hal4KM*) Hal4 proteins fused to GST were grown on YES plates containing 0.1 M NaCl and 2 mM LiCl. Expression of the GST-Hal4 and GST-Hal4KM proteins was confirmed by glutathione-beads precipitation and SDS-PAGE followed by Coomassie blue staining (right panel). (C) Wild-type and a mutant Hal4 lacking the Spc1-binding domain (residues 181-230) were expressed in the *Δhal4* (CA1787) strain using the

pREP1 expression vector (29) with the C-terminal HA epitope, and cell growth was tested on YES plates containing 0.1 M NaCl, 2 mM LiCl and 80 mM $CaCl_2$. Hal4Δ(180-230) complemented the Na^+- and Li^+-, but not Ca^{2+}-, sensitive phenotypes of the Δ*hal4* mutant. The expression of the full-length and Δ(181-230) Hal4 proteins was confirmed by anti-HA immunoblotting (right panels); anti-tubulin staining of the same membrane served as a loading control.

FIG. 6. The cation sensitivity phenotype of the Δ*hal4* mutant is rescued by overexpression of the Trk1 potassium transporter. (A) *trk1*$^+$ was isolated as a multi-copy suppressor of the Δ*hal4* Li^+-sensitivity. Wild-type (KS1598) and Δ*hal4* (CA1787) strains transformed with the empty vector plasmid and the same multi-copy vector carrying the *hal4*$^+$ (pHAL4) or *trk1* (pTRK1) were grown in EMM minimal medium and subjected to the spot test on YES plates with 0.1 M NaCl, 2 mM LiCl or 30 mM $CaCl_2$. The *trk1* genomic fragment in pTRK1 isolated in the multi-copy screen was found to be truncated, expressing Trk1 lacking C-terminal 38 residues. (B) Wild-type (KS1598) and Δ*hal4* (CA1787) strains transformed with the empty pNMT1 expression vector or the same vector to express the full-length (pNMT1-TRK1) and a truncated Trk1 lacking C-terminal 38 residues (pNMT1-TRK1ΔC) were subjected to the spot test as in (A). Expression of the truncated Trk1 efficiently complemented the Δ*hal4* phenotype. (C) Trk1 overexpression does not complement the Δ*spc1* phenotypes. Wild-type (KS1598) and Δ*spc1* (KS1366) strains carrying the empty vector, the *spc1*$^+$ (pSPC1) or *trk1* (pTRK1) plasmids were subjected to the spot test as in (A), using YES plates containing 0.1 M NaCl, 4 mM LiCl and 80 mM $CaCl_2$. Overexpression of *trk1* in Δ*spc1* cells from the pTRK1 plasmid was confirmed by Northern blotting (right panel). 25 μg of total RNA from the Δ*trk1* mutant ("Δ"; CA2227) and Δ*spc1* strains carrying the vector or pTRK1 were hybridized with ^{32}P-labeled *trk1*$^+$ probe.

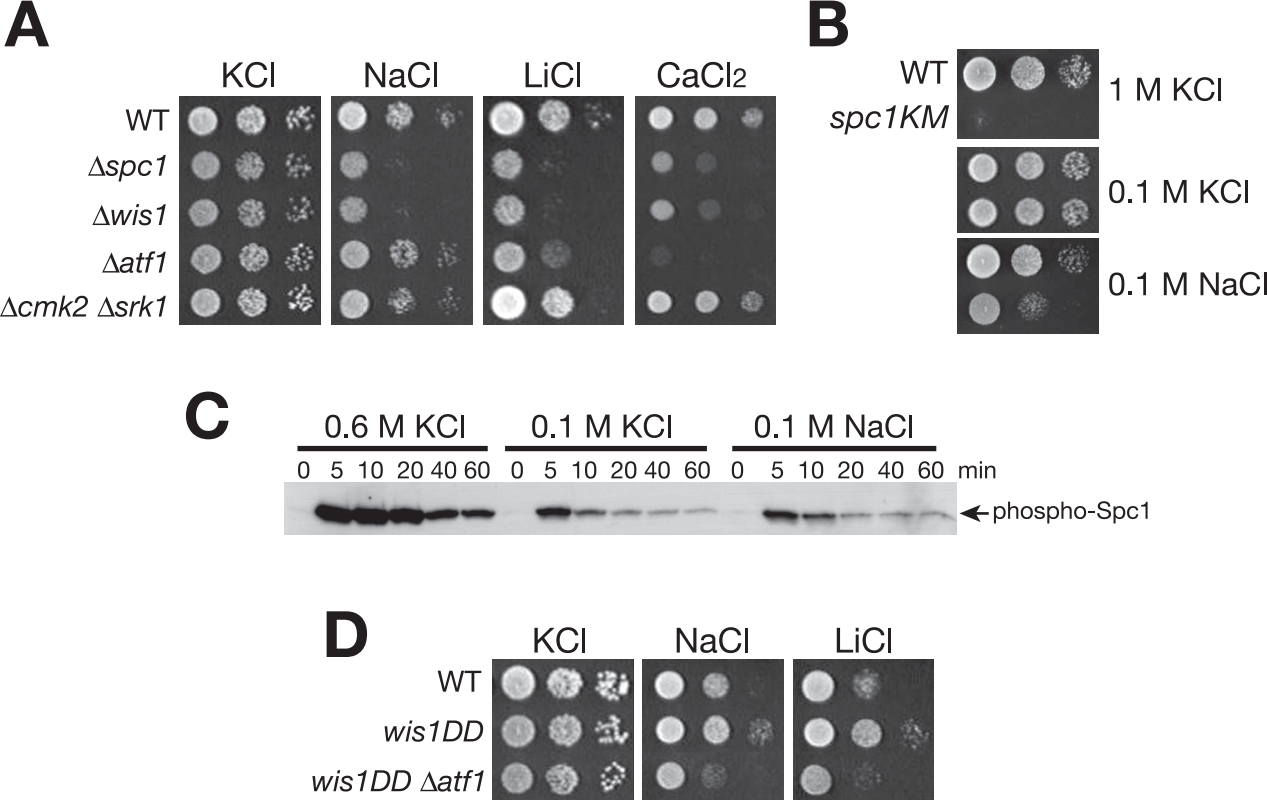
FIG. 7. (A) The growth defect of the Δ*hal4* mutant under K^+-limited conditions. Wild-type (KS1598) and *Δhal4* (CA1787) strains were grown at 30°C in SD medium supplemented with

0.2M KCl to the mid-log phase. Cells were washed by SD medium and then diluted at time 0 to 0.2 OD_{600} in SD medium with (+ KCl) or without 0.2 M KCl, and their growth at 30^{o}C was monitored. (B) Growth of wild-type (KS1598), Δ*hal4* (CA1787), Δ*trk1* (CA2227) and Δ*spc1* (CA1366) cells were examined by the spot test on YES agar plates containing 0.1 M NaCl and 2 mM LiCl, with (+ KCl) or without supplement of 50 mM KCl. The cation sensitivity phenotypes of the Δ*hal4* and Δ*trk1* mutants were completely rescued by increased K^+ in the growth medium. The Δ*spc1* cation sensitivities were also partially complemented by addition of KCl.

TABLE 1. *S. pombe* strains used in this study

Strain	Genotype[a]	Source or reference
PR109	*h⁻ ura4-D18*	Laboratory stock
JM544	*h⁻ ura4-D18 wis1::ura4⁺*	Laboratory stock
KS1366	*h⁻ ura4-D18 spc1::ura4⁺*	(50)
KS1376	*h⁻ ura4-D18 spc1:HA6H*(*ura4⁺*)	(50)
KS1497	*h⁻ ura4-D18 atf1::ura4⁺*	(51)
KS1598	*h⁻*	Laboratory stock
KS2012	*h⁻ ura4-D18 cmk2:myc*(*ura4⁺*)	Laboratory stock
KS2081	*h⁻ ura4-D18 wis1DD:myc*(*ura4⁺*)	(55)
CA96	*h⁻ ura4-D18 pyp1::ura4⁺*	(31)
CA363	*h⁻ ura4-D18 wis1DD:myc*(*ura4⁺*) *atf1::ura4⁺*	Laboratory stock
CA1787	*h⁻ ura4-D18 hal4::ura4⁺*	This study
CA1817	*h⁻ ura4-D18 hal4:GFP*(*ura4⁺*)	This study
CA1992	*h⁻ ura4-D18 spc1::ura4⁺ hal4::ura4⁺*	This study
CA1998	*h⁻ ura4-D18 hal4:myc*(*ura4⁺*)	This study
CA2021	*h⁻ ura4-D18 spc1::ura4⁺ hal4:myc*(*ura4⁺*)	This study
CA2227	*h⁻ trk1::kanR*	This study
CA2282	*h⁺ trk1::kanR trk2::kanR*	This study
CA2685	*h⁻ ura4-D18 arg3-D4 cmk2::ura4⁺ srk1::arg3⁺*	Janet Quinn
CA3079	*h⁻ ura4-D18 hal4:GFP*(*ura4⁺*) *spc1:myc*(*ura4⁺*)	This study
CA3081	*h⁻ spc1KM*	This study

[a]All strains are *leu1-32*.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Δatf1
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0.1 M KCl
0.1 M NaCl
C
0.6 M KCl
0.1 M KCl
0.1 M NaCl
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LiCl
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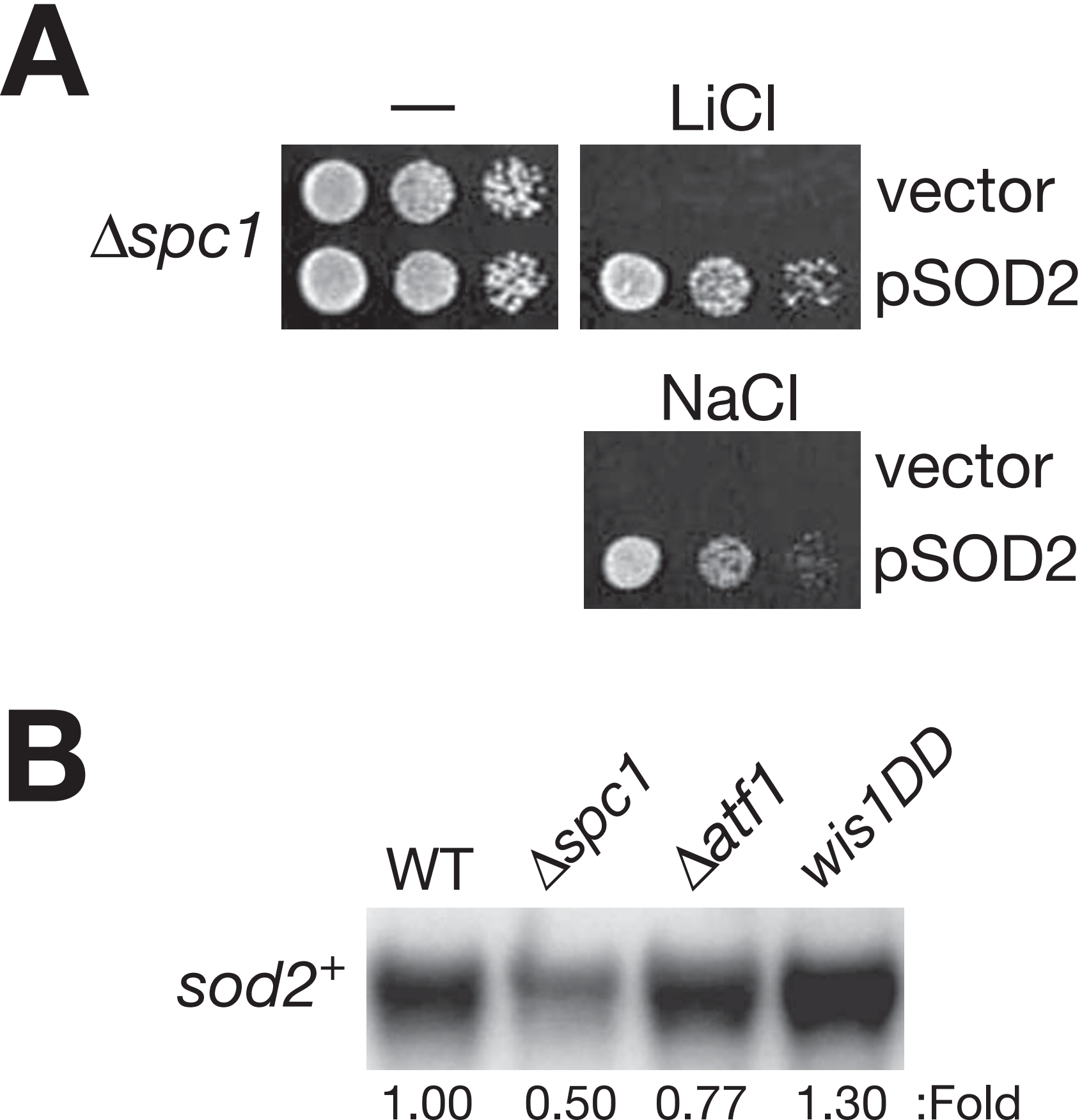
wis1DD
wis1DD Δatf1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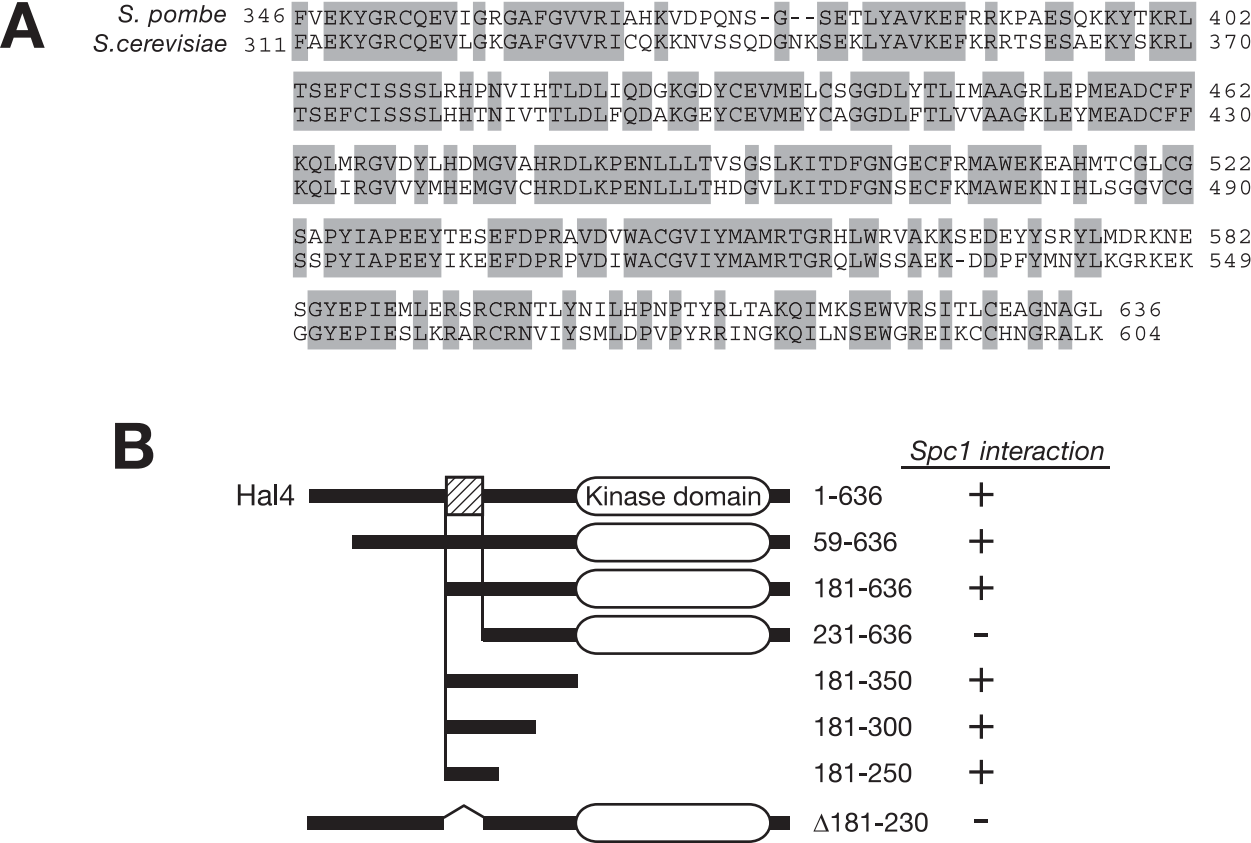
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Spc1 interaction
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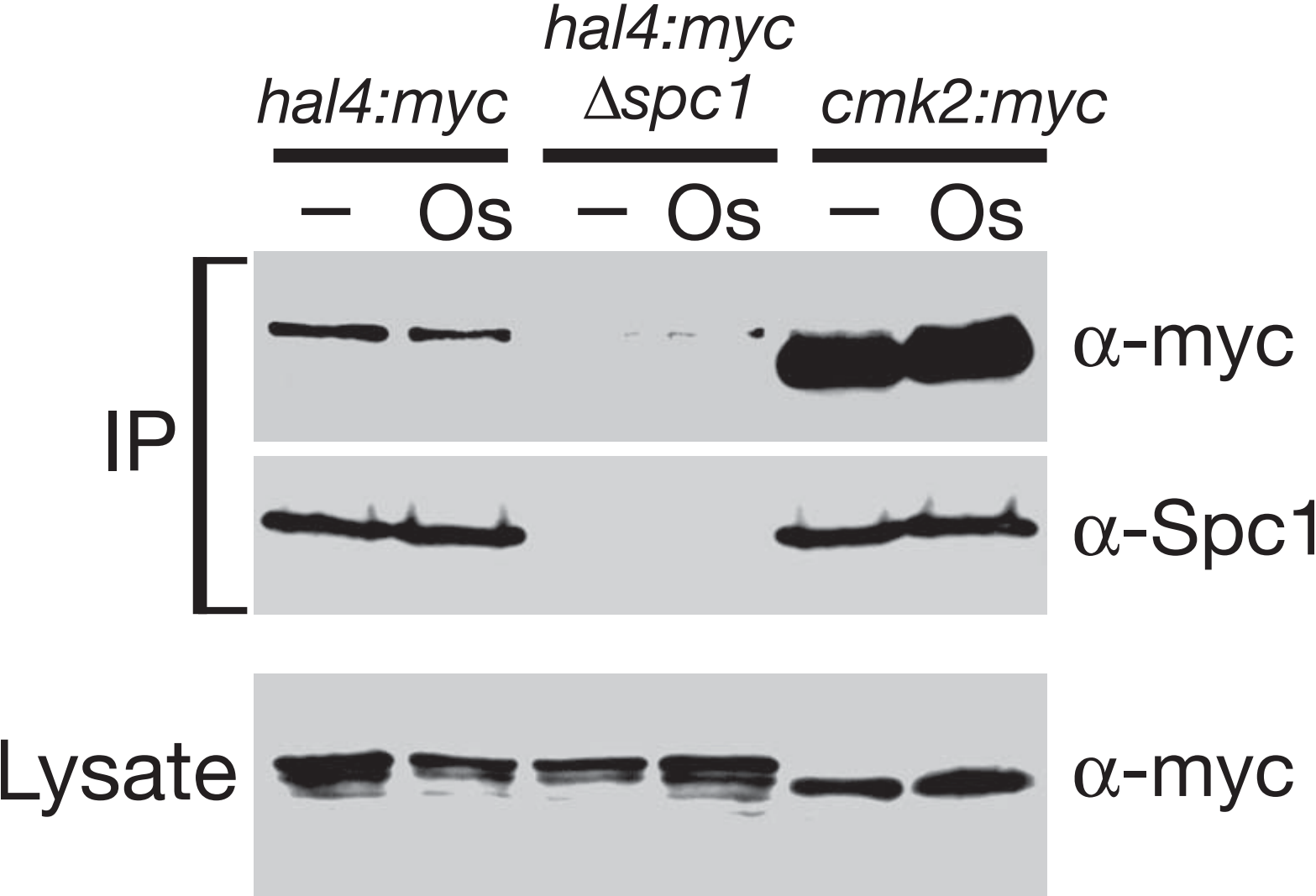
Δ181-230 -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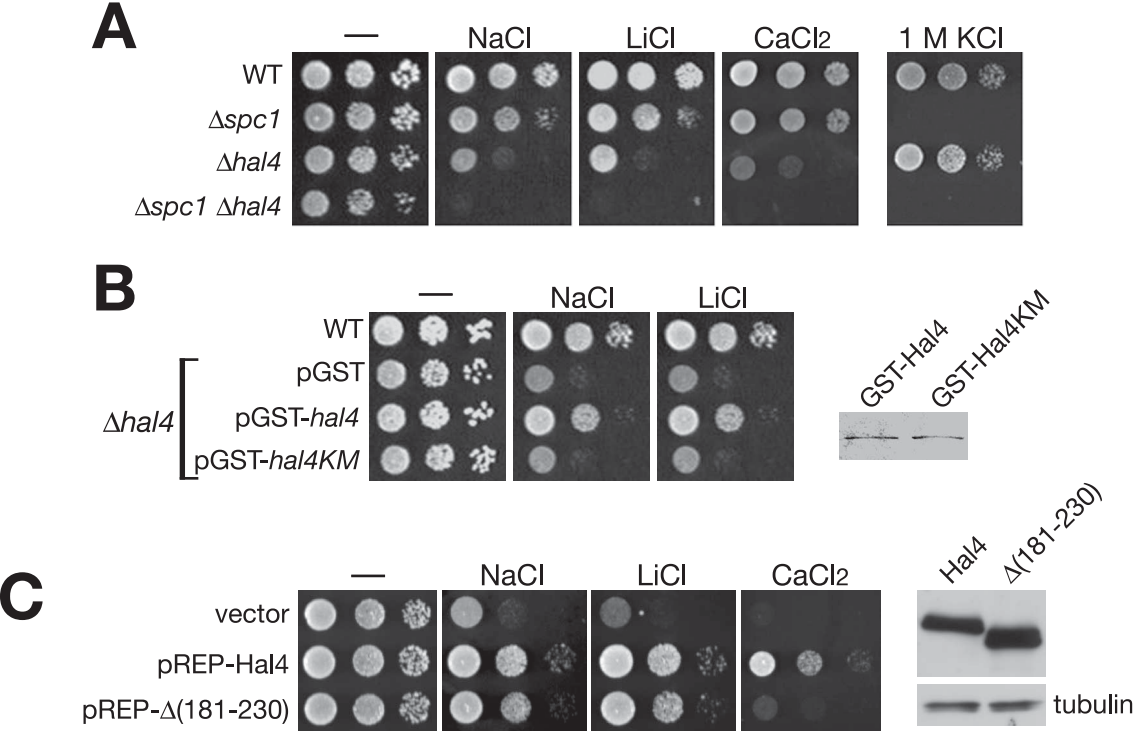
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GST-Hal4KM
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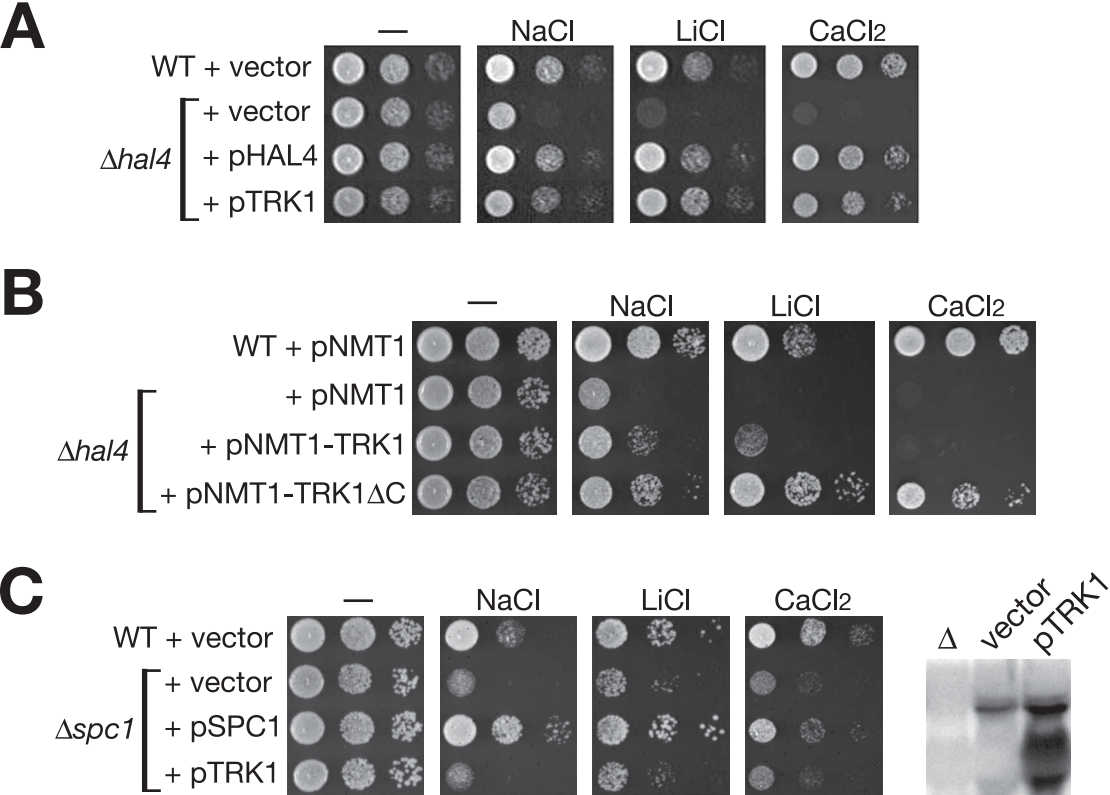
Δ(181-230)
tubulin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Δhal4
+ pNMT1
+ pNMT1-TRK1
+ pNMT1-TRK1ΔC
C
—
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CaCl2
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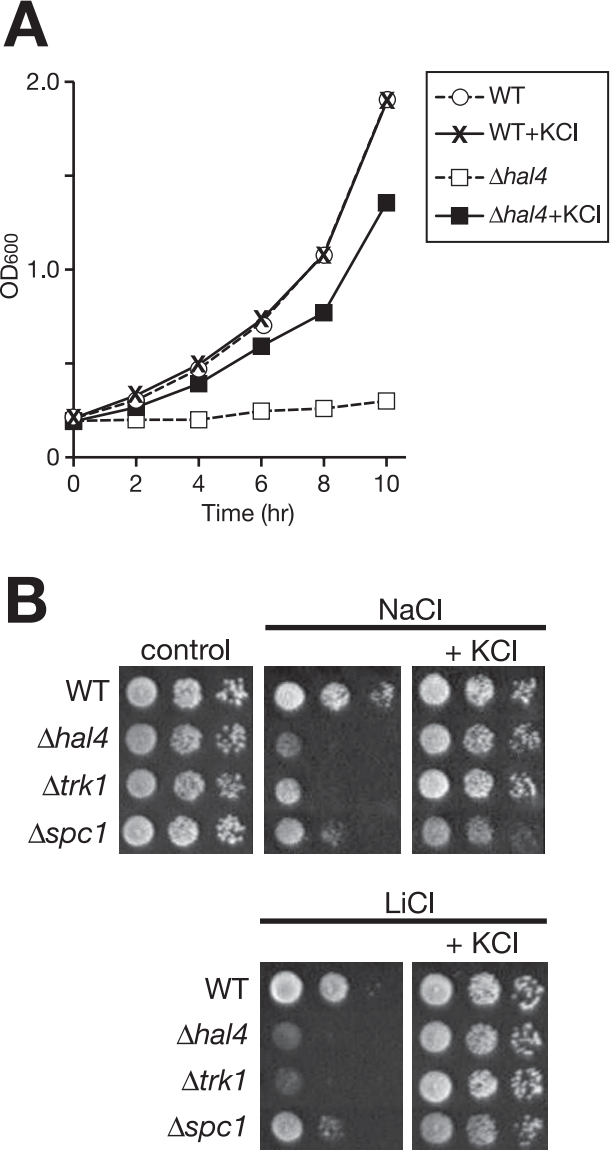
+ pSPC1
+ pTRK1
Δ
vector
pTRK1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6
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10
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WT+KCl
Δhal4
Δhal4+KCl
B
NaCl
control
+ KCl
WT
Δhal4
Δtrk1
Δspc1
LiCl
+ KCl
WT
Δhal4
Δtrk1
Δspc1