Fission Yeast Homolog of Neuronal Calcium Sensor-1 (Ncs1p) 
Regulates Sporulation and Confers Calcium Tolerance*

Nobuko Hamasaki-Katagiri‡, Tatiana Molchanova‡, Kazuyo Takeda¶, and James B. Ames‡**

‡Center for Advanced Research in Biotechnology, University of Maryland Biotechnology Institute, Rockville, MD  20850. ¶Pathology Core, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892.

Running title:  S. pombe Ncs1p regulates sporulation and confers Ca$^{2+}$ tolerance

*This work was supported by NIH Grant EY12347 and a Beckman Foundation Young Investigator Award to J.B.A.

**Corresponding author:
Dr. James B. Ames  
Center for Advanced Research in Biotechnology  
9600 Gudelsky Drive 
Rockville, MD 20850  
Phone:  (301) 738-6120  
FAX:  (301) 738-6255  
e-mail:  james@carb.nist.gov
Summary: The neuronal calcium sensor (NCS) proteins (e.g. recoverin, neurocalcins, and frequenin) are expressed at highest levels in excitable cells and some of them regulate desensitization of G protein-coupled receptors. Here we present nuclear magnetic resonance (NMR) analysis and genetic functional studies of an NCS homolog in fission yeast (Ncs1p). Ncs1p binds three Ca$^{2+}$ ions at saturation with an apparent affinity of 2 µM and Hill coefficient of 1.9. Analysis of NMR and fluorescence spectra of Ncs1p revealed significant Ca$^{2+}$-induced protein conformational changes indicative of a Ca$^{2+}$-myristoyl switch. The amino-terminal myristoyl group is sequestered inside a hydrophobic cavity of the Ca$^{2+}$-free protein and becomes solvent-exposed in the Ca$^{2+}$-bound protein. Subcellular fractionation experiments showed that myristoylation and Ca$^{2+}$-binding by Ncs1p are essential for its translocation from cytoplasm to membranes. The ncs1 deletion mutant (ncs1Δ) showed two distinct phenotypes: nutrition-insensitive sexual development and a growth defect at high levels of extracellular Ca$^{2+}$ (0.1 M CaCl$_2$). Analysis of Ncs1p mutants lacking myristoylation (Ncs1p$^{G2A}$) or deficient in Ca$^{2+}$ binding (Ncs1p$^{E84Q,E120Q,E168Q}$) revealed that Ca$^{2+}$ binding was essential for both phenotypes while myristoylation was less critical. Exogenous cAMP, a key regulator for sexual development, suppressed conjugation and sporulation of ncs1Δ, suggesting involvement of Ncs1p in the adenylate cyclase pathway turned on by the glucose sensing G protein coupled receptor, Git3p. Starvation-independent sexual development of ncs1Δ was also complemented by retinal recoverin, which controls Ca$^{2+}$-regulated desensitization of rhodopsin. In contrast, the Ca$^{2+}$ intolerance of ncs1Δ was not affected by cAMP or recoverin, suggesting that the two ncs1Δ phenotypes are mechanistically independent. We propose that Schizosaccharomyces pombe Ncs1p negatively regulates sporulation perhaps by controlling Ca$^{2+}$-dependent desensitization of Git3p.
Calcium ion \((Ca^{2+})\) regulates physiological processes in the fission yeast, *Schizosaccharomyces pombe* (reviewed in (1-3)), as it does in other eukaryotic cell types (reviewed in (4)). The fission yeast genome encodes ion channels (5,6) and pumps (7-9) homologous to those involved in regulated \(Ca^{2+}\) entry and maintenance of \(Ca^{2+}\) homeostasis in mammalian cells. In all eukaryotic cells, the effects of changes in intracellular \(Ca^{2+}\) levels are mediated primarily by \(Ca^{2+}\)-binding proteins that belong to the EF-hand superfamily (10). In *S. pombe*, a total of 23 genes specify EF-hand-containing \(Ca^{2+}\)-binding proteins as exemplified by *cam1*, calmodulin (SPAC3A12.14) (11); *fim1*, fimbrin (SPBC1778.06c) (12); *plc1*, phospholipase C (SPAC22F8.11) (13); *rlc1*, myosin light chain II (SPAC926.03) (14); *cdc31*, caltractin (SPCC1682.04) (15); and *cdc4*, myosin light chain I (SPAP8A3.08) (16) (Sanger Institute GeneDB). The open reading frame, SPAC18B11.04 (Genbank AC# Z50728) specifies a hypothetical EF-hand protein (Ncs1p, SWISS PROT: Q09711) that is highly homologous to a class of neuronal calcium-sensing proteins (NCS family (17)) expressed mostly in the central nervous system and in other excitable cell types (18,19).

The primary sequence of *S. pombe* Ncs1p demonstrates its homology to the NCS branch of the EF-hand superfamily of \(Ca^{2+}\)-binding proteins (Fig. 1) (20,21). Recoverin, the most intensively studied NCS protein, serves as a \(Ca^{2+}\) sensor in retinal rod and cone cells where it controls the desensitization of rhodopsin by inhibiting rhodopsin kinase only at high \(Ca^{2+}\) levels (22-25). The NCS family also includes neuronal \(Ca^{2+}\) sensors such as neurocalcin (26), hippocalcin (27), and frequenin (28), as well as the budding yeast homolog, Frq1 (29). All members of the NCS family are myristoylated and possess four EF-hands, although the first EF-hand motif (EF-1) contains substitutions that prevent \(Ca^{2+}\)-binding at this site (Fig. 1). In recoverin, substitutions in EF-4 also prevent \(Ca^{2+}\) binding, and hence only EF-2 and EF-3 are functional (30). Frq1, frequenin and neurocalcin contain three occupied \(Ca^{2+}\)-binding sites (EF-2, EF-3 and EF-4) in their atomic resolution structures (31-33). The *S. pombe* Ncs1p shares highest sequence identity with Frq1 and frequenin (60%), but is also highly similar in sequence with mammalian recoverin (46% identity). Interestingly, structurally important amino acids in recoverin appear invariant in *S. pombe* Ncs1p,
which are not conserved in *S. cerevisiae* Frq1 (see highlighted residues in Fig. 1). Moreover, recoverin is significantly closer in sequence to Ncs1p (46% identity) than to Frq1 (35% identity). Hence, recoverin appears to be structurally and evolutionarily more similar to *S. pombe* Ncs1p than it is to Frq1 of budding yeast.

The three-dimensional structures of myristoylated recoverin have been determined by nuclear magnetic resonance (NMR) spectroscopy (30,34). A striking feature of these structures is a large Ca\(^{2+}\)-induced conformational change. In the Ca\(^{2+}\)-free state, the myristoyl group is buried inside the protein and not exposed to solvent. Binding of Ca\(^{2+}\) to recoverin leads to extrusion of its myristoyl group and to a large rotation of the two domains of the protein such that many hydrophobic residues are exposed. The Ca\(^{2+}\)-induced exposure of the myristoyl group, termed the calcium-myristoyl switch, enables recoverin to bind to membranes only at high Ca\(^{2+}\) levels (35,36).

Given the similarities of Ncs1p to the NCS family, it was of interest to assess whether this ancestral homolog displays a Ca\(^{2+}\)-dependent myristoyl switch and to characterize its physiological role in fission yeast. Here we combine NMR spectroscopy, biochemical analysis and yeast genetics to determine the Ca\(^{2+}\) and membrane binding properties, Ca\(^{2+}\)-induced protein structural changes, and biological function of Ncs1p.
EXPERIMENTAL PROCEDURES

Strains and media S. pombe strains constructed in this study were derived from either wildtype KGY425, KGY461, KGY553, KGY554 or SP870 (Table 1). Standard culture media and genetic manipulation techniques were employed (37). For vegetative growth of S. pombe, rich medium (YES) and synthetic minimum medium (EMM) were used. ME solid medium was used for conjugation and sporulation. Nutritional supplements were added as needed. In order to maintain strains bearing S. pombe plasmid with LEU2 marker and nmt1 promoter, leucine was omitted from the supplements in EMM media (EMM-L) and 2 µM thiamine was added. To see starvation-independent spore formation, EMM media containing a final concentration of 3% glucose, equivalent concentration to rich medium, was used. For the calcium sensitivity assay, CaCl₂ was added to YES media at a final concentration of 0.1 M before autoclaving. The deletion mutant strain, bearing a plasmid based on S. pombe expression vector pREP1 (LEU2⁺ nmt1 promoter), was grown on EMM-L minus thiamine to induce the nmt1 promoter before being streaked on YES containing 0.1 M CaCl₂.

Ploidy was confirmed by the color of colonies on an agar medium containing 0.0005% Phloxin-B (YEP), by the color of colony on agar medium containing low concentration of adenine (YES with no adenine added), and by the cell size and presence of azygotic sporulation. Routine microscopic observation was performed by Olympus BX51 fluorescence microscope equipped with a CCD camera (SPOT-RT Slider, Diagnostic). Live cells were stained with DAPI by mixing equal volume of cell suspension and 50 µg/ml DAPI solution. Live cells were also examined by confocal microscopy TCS-SP with DMRE (Leica, Heidelberg, Germany), equipped with Ar⁺ laser source. Excitation for GFP is 488 nm and emission is 500-575 nm. Twenty to thirty slices of optical sectioning were made by serial scanning and images were stored for further 3D reconstruction. Three-dimensional animation movies were generated from these data (see supplemental material).

Cloning of ncs1 and plasmid construction Standard protocols were used (38) and Escherichia coli DH5α was used as a host for all plasmid manipulations. Nucleotide sequences
were determined after gene cloning and each mutagenesis by Dye Terminator Cycle Sequencing by using ABI 373 or 3100 sequencer (Perkin-Elmer).

The ncs1 gene was cloned by RT-PCR; first, total RNA was extracted from S. pombe canonical wild type strain 972 by Trizol Reagent (Gibco BRL-Invitrogen) followed by reverse transcription. Resultant cDNA mixture was served as a template in the PCR and upstream and downstream primers, 5'-AAGGTACCGT CGACATATGG GAAAATCACA ATCAAAAATGG TCTCAAGATC AACTCC-3' and 5'-AATCTAGAGG ATCCTTCATT ATCCTT CATT ATATTAAAAA ATGTAAT AAA AGGCAAATG TAC-3’ respectively, were used. An approximately 0.8 kb PCR product was first cloned into pBlueScript KSII(+) (Stratagene) at KpnI/XbaI sites yielding pBSII-ncs1. The nucleotide sequence of ncs1 ORF was confirmed and this plasmid was used as a template for all the mutagenesis. Triple EQ mutations (3EQ; E84Q,E120Q,E168Q) that abolished Ca2+ binding at EF-2, EF-3 and EF-4 were created by QuikChange Site-Directed Mutagenesis Kit (Stratagene). The G2A mutation was performed by standard PCR using Pfu polymerase (Stratagene).

The multicopy S. pombe expression vector pREP12) was used to express ncs1, ncs1G2A, ncs13EQ, bovine recoverin cDNA (Reco) or S. cerevisiae FRQ1 gene. In order to construct these plasmids, ncs1 and its mutant genes as well as Reco and FRQ1 in pTIS1DT (39) were all subcloned into pREP1 at either NdeI/XmaI site or NdeI/BamHI site.

To construct fusion proteins of Ncs1p and its unmyristoylated mutant with EGFP (enhanced green fluorescence protein; F64L S65T, mut1 of Cormack (40)), the ncs1 and ncs1G2A genes were modified at their stop codons and ligated into pREP42EGFP-C2) at NdeI/XmaI site. For protein expression in E. coli, the ncs1 ORF from pBSII-ncs1 was subcloned into pPET11a (Novagen) at NdeI/BamHI sites.

Gene disruption of ncs1 allele Deletion mutant allele, ncs1Δ::his3, was produced as shown in Fig. 6 by one-step gene disruption. The coding region of ncs1 was replaced with a his3 gene cassette through homologous recombination in wild-type diploid NHSP001D. The his3 gene cassette was made by PCR using pAF12) as a template and 5'-GGTTTTTTAA AGACTGCCCC
TCTGGCCATT TGAATAAGTC TGAATTCAG AAAATCTATA AACAATTTTT
CCCATTCGGT GATCCCTTTC AACGTTTTC TTTACT-3’ and 5’- AACCAATCCA
TCGTATAAAG AAAGGGCTGA CACGATAGTC GGATCACGTT TGGACCCTTC
GCAGAATTCC TCAAGTGTCA GTTGCTCTAT GCAAAGCTAA CGAAT-3’ as upstream
and downstream primers, respectively. The his3+ haploid NHP013 was obtained from resultant
heterozygous diploid NHP009D by random sporulation (41). The isogenic strains NHP57-60 were
obtained by back-crossing NHP013 with KGY461 (Table 1). Homothallic deletion mutant
NHP033 was obtained using the same cassette and SP870 was used as a host strain.

The chromosomal ncs1-GFP tagged allele (ncs1+-GFP::ura4+) was constructed by the same
method using GFP::ura4+ cassette delivered from pCS2pkSu2 and KGY425 as a host strain.
Resultant NHP002 was then back-crossed with KGY554 to obtain NHP078 (Table 1). Ncs1p-GFP
was confirmed to be as functional in nutrition dependent sporulation as wildtype.

Preparation and purification of recombinant myristoylated Ncs1p To prepare recombinant
myristoylated Ncs1p protein uniformly labeled with nitrogen-15 and/or 13C14 myristate, the protein
was expressed in E. coli strain BL21(DE3) (Novagen) carrying the pET11a vector harboring the
ncs1 coding sequence and co-expressing a yeast N-myristoyltransferase (42), grown in M9 minimal
medium containing (15N)-NH4Cl according to well-established procedures (43,44). Unlabeled or
(13C14)-myristic acid (5 mg/l) was added to the medium 0.5 hr before induction of protein
expression. Labeled myristoylated Ncs1p protein was purified from the soluble fraction of a
bacterial cell lysate using hydrophobic interaction chromatography as described previously for
recoverin and frequenin (35,45). Peak fractions were concentrated to 5 ml and subjected to size-
exclusion chromatography (Sephacryl S-100, Pharmacia) in buffer B (1 mM dithiothreitol, 2 mM
CaCl2, 50 mM HEPES, pH 7.4). Final purity was greater than 98%, as judged by SDS-PAGE
(supplemental data5). Electrospray-ionization mass spectrometry indicated that at least 85% of the
recombinant protein was properly myristoylated.

NMR spectroscopy Samples for NMR analysis were prepared by dissolving 15N-labeled
Ncs1p (0.5 mM) in 0.5 ml of a 95% H2O/5% 2H2O solution containing 10 mM (2H2)-imidazole (pH
7.2), 10 mM (2H10)-dithiothreitol and either 1 mM EDTA (Ca2+-free) or 5 mM CaCl2 (Ca2+-bound). All NMR experiments were performed at 35°C on a Bruker DRX-500 or DRX-600 spectrometer equipped with a four-channel interface and a triple-resonance probe with triple-axis pulsed field gradients. The 15N-1H HSQC spectra (see Fig. 3) were recorded on a sample of 15N-labeled Ncs1p (in 95% H2O, 5% 2H2O). The number of complex points and acquisition times were: 256, 180 ms (15N (F1)); and, 512, 64 ms (1H (F2)). The 13C(F1)-edited, 13C(F3)-filtered NOESY-HMQC spectra (see Figure 4) were recorded on a sample of unlabeled Ncs1p protein containing a uniformly 13C-labeled myristoyl group (46).

**Binding of 45Ca2+.** 45Ca2+ radioactive isotope (calcium-45, calcium chloride in aqueous solution, specific activity = 850 mCi/mL, Amersham Pharmacia Biotech) was used to quantitate the binding of Ca2+ to Ncs1p. 45Ca2+ binding to Ncs1p was measured as the protein-bound radioactivity retained after ultrafiltration using a previously described procedure (47) based on the original method of Paulus (48). The buffer used in the Ca2+ titration (25 mM HEPES, 0.1 M KCl, 1 mM dithiothreitol, pH 7.4) and protein samples were decalcified by treatment with Chelex resin (Bio-Rad Laboratories) using the batch method. A Centricon-10 concentrator (10 kDa cutoff, 2 mL sample compartment, Millipore Corp.) used in the titration was pretreated to remove contaminating Ca2+. The lower chamber was rinsed with 0.1 M HCl followed by several rinses with decalcified buffer. The concentrator membrane was decalcified by rinsing with 5% NaHCO3 followed by several rinses with decalcified buffer. A decalcified protein sample (1.5 mL, 70 μM) was placed into the sample compartment, and 12 μL of 0.25 mM 45Ca2+ solution (2.6 μCi) was added. The sample was carefully mixed and centrifuged (2300 rpm, 2 min) using a tabletop centrifuge (Beckman Model TJ-6), forming 25 μL of filtrate. The filtrate was returned to the sample chamber, mixed and centrifuged a second time to minimize any dead volume. The radioactivity of 10 μL of the filtrate (free Ca2+) and an equal volume of the protein sample (total Ca2+) were determined by liquid scintillation counting (Liquid Scintillation Analyzer, Packard Instrument Co.). Aliquots of nonradioactive Ca2+ were added serially to the protein sample to adjust the total Ca2+ concentration throughout the titration (5.5, 15.7, 28.7, 52.5, 78.7, 131, 180, 240, 300, 400, 500, 600 μM), and the
above centrifugation procedure was repeated in triplicate for each point in the titration. The free and bound Ca\(^{2+}\) concentrations at each point in the titration were calculated from the measured radioactivity as described previously (29) and fractional saturation was plotted as a function of free Ca\(^{2+}\) concentration (see Table 2 and Fig. 2).

**Subcellular fractionation and Western blot analyses.** *S. pombe* NHP057 (ncs1\(\Delta\)) bearing pREP42-ncs1-EGFP or pREP42-ncs1\(^{G24}\)-EGFP were grown for 20 hrs after induction of nmt1 promoter (by decreasing the thiamine concentration in the media from 2 \(\mu\)M down to 0.05 \(\mu\)M) to mid-log phase and rinsed in buffer (EB; 15 mM KCl, 10 mM HEPES-KOH pH 7.8, 3 mM DTT, 1 mM PMSF) containing either 1 mM CaCl\(_2\) or 10 mM each of EDTA and EGTA. Cells were then lysed by glass beads in the same buffer containing either 0.2 mM CaCl\(_2\) or chelating reagents (5 mM each of EDTA and EGTA) and the cell-free extract (lysate) was obtained after centrifugation at ca. 500\(g\) for 5 min at 4 \(^\circ\)C. Protein concentration of the lysate was determined, and each lysate was adjusted with its respective buffer to the same final concentration. Part of these cell-free extracts, 100 \(\mu\)l each, were subjected to centrifugation for 30 min at ca.16,000\(g\) resulting in the pellet and supernatant fractions. The pellet was resuspended with an equal volume of the appropriate lysis buffer. The lysate, supernatant fractions and resuspended pellets were subjected to SDS-PAGE (15% acrylamide) followed by Western blot analysis with anti-GFP antibody (mouse polyclonal antibody, Clontech). Signals were detected using chemiluminescent substrate (Super Signal West Dura, Pierce). In case of experiments in Fig. 9C (right panel), cells were lysed in EB buffer without any additional CaCl\(_2\) or chelating reagents and whole lysate was loaded on the gel.

**Assay for mating, sporulation and germination efficiency.** To score the “color test” for diploid stability shown in Figure 7A, pairs of fresh haploid strains from NHP057-60 were mixed on mating/sporulation plate (ME) and diploid cells from each pair were isolated on EMM lacking adenine (EMM-A) containing 3% glucose. More than four colonies from EMM-A were periodically streaked on YES+low adenine (no adenine added) plate. After two or more days, white colonies (representing diploid cells) and pink colonies (representing haploid cells) appeared. Stability of diploid cells was quantified as the percentage of white colonies counted as a function
of time. Two independent experiments were performed: Experiment #1 quantified the percentage of white colonies from cells grown on a low adenine plate for 2 days, and Experiment #2 quantified from cells grown for four days.

The measurement of starvation independent conjugation/spore formation was also performed by observation of morphological change using the homothallic strain NHP033 (ncs1Δ) bearing various expression plasmids: empty vector pREP1, pREP1-ncs1, pREP1-ncs1G2A, pREP1-ncs1E84Q,E120Q,E168Q, pREP1-recoverin or pREP1-FRQ1 (Table 3). Homothallic (h90) strains change their mating type periodically and start a series of sexual development upon change of nutritional condition. In this case, sexual development was triggered by temperature shift because ncs1 mutant was less sensitive to nutritional starvation. Cells from a single colony isolated from minimal medium plate (EMM) lacking leucine and containing 3% glucose and thiamine were grown in liquid media (EMM-L) at 37 °C, to avoid sporulation, until OD600 reached between 1-2. Cells were resuspended in the same media giving an OD600 of 1.0 and incubated at 25 °C without shaking. Alternatively, a drop of cell suspension was placed on EMM-L plate and incubated at 25 °C. Morphology was observed periodically using a microscope. Individual cells and asci were identified and counted. Typically, 600 cells were counted.
RESULTS

Cloning of ncs1. Primers for RT-PCR of *S. pombe* gene (SPAC18B11.04) were designed according to the sequence of chromosome I cosmid c18B11 (Genbank AC# Z50728), and a cDNA (819 base pairs) was cloned from canonical wild type fission yeast, same as a strain used in genome sequencing, as described in Experimental Procedures. The open reading frame consists of four exons and encodes a protein of 190 amino acids that is highly homologous to neuronal calcium sensor (NCS) proteins (17). Hence, this gene has been named, *ncs1*.

Equilibrium Ca$^{2+}$-binding Measurements. Ncs1p contains four EF-hand Ca$^{2+}$-binding motifs (Fig. 1). The first EF-hand (EF-1) of Ncs1p contains substitutions (Lys36, Cys38 and Pro 39) that would be expected to disrupt the structure of this binding loop and prevent Ca$^{2+}$ binding to EF-1, as seen in the crystal structures of recoverin, frequenin and neurocalcin (32,33,49). In recoverin, the disabling of Ca$^{2+}$ binding to EF-1 seems to be important for its calcium-myristoyl switch mechanism because residues of EF-1 in Ca$^{2+}$-free recoverin make physical contact with the myristoyl group (30). Likewise, the presence of residues that potentially disable Ca$^{2+}$ binding to EF-1 suggests that Ncs1p may possess a similar calcium-myristoyl switch mechanism. In contrast to recoverin, in which EF-4 is also disabled by substitutions incompatible with Ca$^{2+}$ binding, the remaining motifs in Ncs1p (EF-2, EF-3 and EF-4) are good matches to the consensus and are expected to bind Ca$^{2+}$. To quantitate the number of ions that bind at saturation, direct measurements of Ca$^{2+}$ binding were performed on samples of recombinant, myristoylated Ncs1p purified from bacteria.

Equilibrium measurements of $^{45}$Ca$^{2+}$ binding were conducted on Nes1p (Fig. 2 and Table 2). At saturation, three Ca$^{2+}$ bind to the protein, consistent with the view that EF-1 is disabled, whereas EF-2, EF-3 and EF-4 are functional. The fractional saturation (Y), which can be obtained from the same data, can be represented by the Hill equation,
where $[\text{Ca}^{2+}]$ is the free Ca$^{2+}$ concentration, $K_d$ is the apparent dissociation constant, and $a$ denotes the Hill coefficient. The Ca$^{2+}$ binding isotherm for Ncs1p (Fig. 2) is best fit by the Hill equation using the parameters, $K_d = 2.1$ $\mu$M and $a = 1.9$. A Hill coefficient of nearly two suggests that two or more Ca$^{2+}$ bind cooperatively to the myristoylated protein. The positive cooperativity of Ca$^{2+}$ binding suggests that Ncs1p undergoes a concerted protein conformational change like recoverin’s Ca$^{2+}$-myristoyl switch (50).

**Structural Studies using NMR.** The above analysis of Ca$^{2+}$ binding to Ncs1p suggests two allosteric states of the protein. To test whether Ncs1p actually undergoes Ca$^{2+}$-induced structural transitions, we collected two-dimensional NMR spectra ($^1$H-$^{15}$N HSQC) of uniformly $^{15}$N-labeled Ca$^{2+}$-free and Ca$^{2+}$-bound forms of myristoylated Ncs1p (Fig. 3). Peaks in each spectrum represent main chain and side-chain amide protons that serve as fingerprints of overall conformation. The NMR spectrum of Ca$^{2+}$-free Ncs1p exhibited many sharp and highly resolved peaks (Fig. 3A). The number of observed peaks (215) was, as expected, very close to the total number of amide protons in the protein (190 main chain + 32 side chain = 222 amide protons). The sharpness of the peaks and uniform peak intensities indicated that Ca$^{2+}$-free, myristoylated Ncs1p adopts a stable three-dimensional fold and is monomeric in solution, similar to the NMR spectrum and structure of Ca$^{2+}$-free recoverin that is highly stabilized by a sequestered myristoyl group (34). Our preliminary structural analysis of Ncs1p suggests that the amino-terminal myristoyl group is also sequestered inside a hydrophobic cavity located primarily in the N-terminal domain of the protein (see below).

In contrast to Ca$^{2+}$-free Ncs1p, the NMR spectrum of Ca$^{2+}$-bound Ncs1p exhibits very broad and poorly resolved peaks (Fig. 3B). The striking Ca$^{2+}$-induced spectral differences are consistent with large Ca$^{2+}$-induced structural changes in the protein. The intensity of peaks characteristic of the Ca$^{2+}$-bound form saturated upon the addition of 3 molar equivalents of Ca$^{2+}$ to the sample, in good agreement with the stoichiometry of Ca$^{2+}$ binding determined from the equilibrium Ca$^{2+}$-
binding experiments (Fig. 2). The number of observed peaks in the spectrum of Ca\(^{2+}\)-bound Ncs1p was much lower than the expected number of amide groups in the protein. Many of the NMR resonances of Ca\(^{2+}\)-bound Ncs1p were not detected apparently because they were severely broad and weak. The variable range of peak intensities suggested that some of the peaks might be broadened due to self-association of Ncs1p molecules in concentrated solution (1 mM protein concentration) required for NMR. Indeed, dynamic light scattering measurements performed on the solutions used for NMR confirmed that the Ca\(^{2+}\)-bound Ncs1p samples contained a broad distribution of multimeric species with an average molecular weight of ~72 kDa (data not shown). The Ca\(^{2+}\)-induced protein aggregation observed here for Ncs1p might be caused by a solvent-exposed myristoyl group like that of Ca\(^{2+}\)-bound recoverin (30,46).

**NMR Analysis of \(^{13}C\)myristate-labeled Ncs1p.** NMR analysis of fatty acyl chain resonances provides a sensitive probe of the disposition of the covalently attached myristoyl group and its interaction with the protein. Previously, we developed and performed three-dimensional (\(^{13}C\)-filtered NOESY-HMQC) NMR experiments on samples of recoverin that contained a \(^{13}C\)-labeled myristoyl group to selectively probe the chemical environment around the amino-terminal myristoyl group (46,47,51). These studies revealed that the covalently attached fatty acyl chain in recoverin is sequestered in a hydrophobic cavity in the Ca\(^{2+}\)-free protein and that binding of Ca\(^{2+}\) leads to conformational changes that extrude the myristoyl group into solvent. In contrast, similar NMR experiments performed on Frq1 containing a \(^{13}C\)-labeled myristoyl group revealed that the fatty acyl chain is solvent exposed in both the Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms (31). This difference in the disposition of the myristoyl group between Frq1 and Ca\(^{2+}\)-free recoverin might be due to changes in critical residues required for the Ca\(^{2+}\)-myristoyl switch (Fig.1).

Three-dimensional (\(^{13}C/F_1\))-edited and (\(^{13}C/F_3\))-filtered NOESY experiments (46,52) were performed on unlabeled Ncs1p protein containing a \(^{13}C\)-labeled myristate (Fig. 4). These spectra selectively probed atoms of residues in the protein that lie within 5 Å of the labeled C\(_{14}\)-methyl group of the myristoyl chain. If the myristoyl group is sequestered within the protein, then the terminal methyl group of the fatty acyl chain is expected to be very close to atoms of the protein,
resulting in strong off-diagonal cross peaks in the filtered NOESY spectrum. The filtered NOESY spectrum of Ca\(^{2+}\)-free Ncs1p (Fig. 4A) exhibits many dipolar interactions between the myristate C\(_{14}\)-methyl group (F\(_2\) = 16.9 ppm, F\(_1\) = 0.9 ppm) and the protein. Strong off-diagonal cross peaks near 1.0 and 7.0 ppm represent close contacts with aliphatic groups and aromatic side chains, respectively. Therefore, the methyl group of myristate interacts intimately with the Ca\(^{2+}\)-free Ncs1p protein and appears buried in a hydrophobic cavity akin to that of Ca\(^{2+}\)-free recoverin (34), in contrast to the solvent-exposed myristoyl group of Ca\(^{2+}\)-free Frq1 (31).

On the contrary, no off-diagonal cross peaks were observed in the filtered NOESY spectrum of Ca\(^{2+}\)-bound Ncs1p (Fig. 4B), suggesting that the C\(_{14}\)-methyl group of the fatty acyl chain does not interact closely with the Ca\(^{2+}\)-bound Ncs1p protein. Hence, the myristoyl group of Ca\(^{2+}\)-bound Ncs1p appears solvent exposed and undergoes a calcium-induced change in environment as expected for a Ca\(^{2+}\)-myristoyl switch. The Ca\(^{2+}\)-induced exposure of the myristoyl group might serve to anchor the Ncs1p protein to cell membranes only at high Ca\(^{2+}\) levels.

**Subcellular Fractionation of Ncs1p.** To assess whether Ncs1p associates with cell membranes, we examined the distribution of myristoylated and unmyristoylated Ncs1p in the soluble and particulate fractions of whole-cell extracts prepared in the presence and absence of Ca\(^{2+}\) (Fig. 5). For this purpose, either myristoylated Ncs1p or an unmyristoylated mutant (Ncs1p\(^{G2A}\)) was expressed as a GFP fusion protein from plasmids in fission yeast that lacked endogenous protein (ncs1\(^{\Delta}\) mutant, see below). The resulting extracts were separated into an insoluble fraction (pellet) consisting mainly of plasma membranes, and soluble fraction (supernatant) containing most of the cytosolic components (53). When the cell lysate was treated with saturating calcium, about half of myristoylated Ncs1p was observed in the pellet (Fig. 5A, lane 2), indicating that substantial portion of the Ca\(^{2+}\)-bound, myristoylated protein was associated with membrane components. In sharp contrast, when the lysate was treated with calcium chelating agents (EGTA and EDTA), nearly all of the Ca\(^{2+}\)-free, myristoylated protein appeared in the soluble fraction, whereas almost none of it could be found in the pellet fraction (Fig. 5A, lane 5). The same results were obtained when Ncs1p was expressed under control of its endogenous promoter in chromosome (Fig. 5D).
Ca\textsuperscript{2+}-induced membrane binding by the Ncs1p-GFP fusion protein was not due to the attached GFP because GFP itself expressed under the same condition was observed only in the supernatant fraction regardless of calcium level (Fig. 5C, lane 2, 3 and 5, 6).

The subcellular fractionation of the unmyristoylated Ncs1p\textsuperscript{G2A} mutant is presented in Fig. 5B. The unmyristoylated protein was observed in both the soluble and insoluble fractions. The cellular distribution of unmyristoylated Ncs1p was found to be independent of Ca\textsuperscript{2+} level (Fig. 5B, lane 2, 3 and 5, 6), in contrast to the Ca\textsuperscript{2+}-induced membrane binding by myristoylated Ncs1p (Fig. 5A). Taken together, these results indicate that the myristoyl group is essential for Ca\textsuperscript{2+}-induced membrane translocation of Ncs1p, and the Ca\textsuperscript{2+}-induced exposure of the myristoyl group (Fig. 4) may help to anchor the protein to membranes only at high Ca\textsuperscript{2+} levels.

*Deletion mutant of ncs1 exhibits starvation-independent conjugation and sporulation.* The ncs1 open reading frame was disrupted by inserting the his3 marker gene (1.9 kb), deleting the entire EF2 and EF3 coding region (Fig. 6). The deletion allele (*ncs1Δ::his3*) was confirmed by Southern blot analysis. A tetrad dissection of heterozygous diploid cells (*ncs1+/ncs1Δ::his3*) produced haploid deletion mutants that were viable with a normal vegetative growth rate. This is a clear difference from the lethal phenotype of deletion mutant of *S. cerevisiae* homolog, Frq1. The haploid *ncs1Δ* strains did not show any temperature sensitivity at 15 °C or 37 °C and exhibited normal morphology.

During our attempt to isolate *ncs1Δ* haploid cells by conventional tetrad dissection, we found that the heterozygous diploid cells (*ncs1\textsuperscript{+}/ncs1Δ::his3*) were very unstable and sporulated and germinated in nutrient-rich medium. More than half of the *ncs1Δ::his3* diploid cells grown on EMM-A containing 3% glucose spontaneously converted into haploid cells within a few days, whereas wildtype diploid cells remained much more stable during the same period under the same conditions. The relative instability of *ncs1Δ* diploid cells was quantified using a simple color test as illustrated in Fig. 7A and described in Experimental Procedures. The percentage of white colonies (representing diploid cells) was much greater for wildtype (54%) compared to that of heterozygous (8% and 9%) and homozygous (11%) strains of *ncs1Δ::his3* (Fig. 7A). This
phenotype was seen in both heterozygous and homozygous diploid cells and was therefore haploid insufficient. Later, we noticed that freshly prepared heterozygous diploid cells could be isolated temporarily but exhibited diploid instability after only a few days, hereafter referred to as starvation-independent sporulation.

Since sexual development in \textit{S. pombe} involves germination that subsequently follows conjugation and sporulation, the observed diploid instability in \textit{ncs1}∆ could be due to abnormal germination. The germination efficiency of \textit{ncs1}∆ mutant measured by random sporulation method was shown to be similar to that of homozygous wildtype (data not shown). Hence, the \textit{ncs1} deletion does not affect germination efficiency.

The homothallic deletion mutant NHP033 (h\textsuperscript{90} \textit{ncs1}∆) enabled synchronous observation of sporulation/conjugation events and was used to more quantitatively evaluate conjugation and sporulation efficiencies. The percentage of homothallic diploid cells that underwent sporulation in nutrient-rich medium after 24 hrs was significantly greater for \textit{ncs1}∆::\textit{his3} (13 \% zygotic asci) compared to that of wildtype (<1 \%) (Fig. 7B). Similarly, the percentage of conjugation in nutrient-rich medium after 24 hrs was also greater for \textit{ncs1}∆::\textit{his3} (9\% zygotes) compared to that of wildtype (4\%) (Fig. 7B). Hence, the \textit{ncs1} deletion mutant exhibits a phenotype similar to starvation-independent sporulation seen previously in the deletion mutants of \textit{git3} (glucose receptor) and \textit{gpa2} (α-subunit of G protein) (54). In \textit{S. pombe}, sexual development is regulated negatively by a nutritional signal (e.g. glucose or nitrogen) and positively by pheromone. Under normal growth conditions (non-starvation), the nutritional signal represses sexual development through sequential activation of the glucose receptor (\textit{git3}), G protein α-subunit (\textit{gpa2}), adenylate cyclase (\textit{cyr1}) and protein kinase A (\textit{pka1}) in a cascade known as the “adenylate cyclase pathway”. Under nutrition starvation, the adenylate cyclase pathway turns off, causing a drop in the cellular cAMP level, which then triggers conjugation/spore formation (55,56). Sporulation is important for the preservation of species because spores are more durable than vegetative cells and more resistant to environmental stresses.
Starvation-independent conjugation and sporulation of the ncs1Δ mutant was effectively blocked by the addition of exogenous cAMP, suggesting possible involvement of the adenylate cyclase pathway. When cells were incubated in EMM with 10 mM cAMP for more than 24 hrs, both wild type and ncs1Δ mutant cells did not conjugate or sporulate: no zygote formation was seen in 600 cells observed (Fig. 7C). This effect of cAMP suggests that Ncs1p might regulate a G protein cascade upstream of adenylate cyclase. Hence, Ncs1p, like recoverin, may serve to couple calcium cascades and G protein cascades.

Retinal recoverin, S. cerevisiae Frq1 and Ncs1pG2A each complemented the starvation-independent sporulation phenotype of ncs1Δ (see Table 3). The ncs1Δ homozygous diploid strain transformed with a multicopy plasmid (pREP1) carrying ncs1 exhibited a markedly lower percentage of cells that sporulate in rich media (3%, as defined in Exp 1, Table 3) compared to that of a negative control (16.7% for deletion mutant transformed with empty pREP1 vector). The ncs1Δ mutant strain was also transformed with the plasmid overexpressing either Ncs1pG2A (unmyristoylated mutant), Ncs1pE84Q/E120Q/E168Q (Ca2+-binding deficient mutant), bovine recoverin, or S. cerevisiae Frq1. The expression of Ncs1pG2A in the ncs1Δ mutant suppressed spore formation in nutrient-rich media (5.7%), but the Ca2+ binding defective mutant (Ncs1pE84Q/E120Q/E168Q) had less of an effect (11.7%). Hence, Ca2+ binding by Ncs1p is essential for suppressing the onset of sporulation in nutrient-rich media. Recoverin or Frq1 overexpressed in the ncs1Δ mutant also suppressed sporulation in rich media, suggesting that both recoverin and Frq1 can functionally substitute for Ncs1p in fission yeast.

Calcium sensitivity of ncs1Δ mutant. The ncs1 deletion mutant in the vegetative haploid state exhibited markedly lower tolerance for high concentrations of extracellular Ca2+ (Fig. 8). When grown on agar plates, wildtype cells grew at very high concentrations of extracellular Ca2+ in excess of 0.5 M. In contrast, the growth of ncs1Δ mutants was halted in the presence of 0.1 M CaCl2 (Fig. 8A) but could grow at 0.01 M CaCl2 or lower concentrations of extracellular Ca2+ in conventional media (~1 mM). The growth defect at 0.1 M Ca2+ was specific for Ca2+ because ncs1Δ mutants grew in the presence of 0.1 M Mg2+ or 1 M K+. Also, the growth defect at 0.1 M Ca2+ was restored by the addition of exogenous cAMP.
CaCl$_2$ was not rescued by 1.2 M sorbitol (osmotic stabilizer), suggesting that the growth defect was not due to an osmotic impairment. The $ncs1\Delta$ cells that stopped growing by Ca$^{2+}$ treatment for more than 48 hrs were able to start dividing again when placed on nutrient-rich media that lacked any added Ca$^{2+}$. The Ca$^{2+}$ sensitivity test using homozygous and heterozygous diploid strains suggested that this phenotype is recessive; i.e. only homozygotic mutant showed sensitivity against 0.1 M Ca$^{2+}$ (data not shown).

The tolerance for high extracellular Ca$^{2+}$ was restored by overexpressing Ncs1p or Ncs1p$^{G2A}$ in the deletion mutant (Fig. 8B), but not by the Ca$^{2+}$-binding defective triple mutant (Ncs1p$^{E84Q\&E120Q\&E168Q}$). This observation indicates that Ca$^{2+}$ binding to Ncs1p plays a physiological role in fission yeast and Ca$^{2+}$-bound Ncs1p prevents growth arrest that otherwise would occur at high concentrations of extracellular calcium. The Ca$^{2+}$ intolerance of the $ncs1\Delta$ mutant was not rescued by overexpressing either Frq1 or recoverin. The expression levels of Ncs1p, Frq1 and recoverin in the $ncs1\Delta$ cells were all confirmed to be same (data not shown) and therefore, the lack of any complementation was not due to a lack of protein expression. The lack of complementation by Frq1 or recoverin suggests that the Ca$^{2+}$ tolerance may not be due to simple Ca$^{2+}$ buffering because both Frq1 and recoverin bind Ca$^{2+}$ with similar affinity ($K_d = 10^{-6}$ M) as Ncs1p. These data show that Ca$^{2+}$-bound Ncs1p has a specific role in protecting the cell from effects at high Ca$^{2+}$ levels.

The growth arrest at high calcium was not rescued by adding exogenous cAMP (data not shown). The lack of any complementation by cAMP, recoverin or Frq1 suggests that the Ca$^{2+}$ sensitivity $ncs1\Delta$ phenotype is mechanistically distinct from the starvation-independent sporulation phenotype of $ncs1\Delta$. Hence, the two $ncs1\Delta$ phenotypes represent two different and perhaps unrelated biological functions for Ncs1p.

**Cellular localization of Ncs1p-GFP.** The in vivo cellular localization of Ncs1p-GFP fusion protein was monitored using fluorescence microscopy. Fluorescence from Ncs1p-GFP in haploid cells grown in rich media (YES) was seen mostly in the cytoplasm and was excluded from the nucleus (Fig. 9A). It is noteworthy that Ncs1p-GFP was concentrated into punctate dots or short
string-like patterns in the cytoplasm, predominantly along the plasma membrane. The membrane association of Ncs1p-GFP was shown more clearly by confocal microscopy (supplement data). A typical 3D image was made from xy scanning plane of a single cell. Careful analysis of the 3D images revealed that the punctuate localization of Ncs1p-GFP was observed near the surface of the plasma membrane with few dots located inside the bulk of the cytoplasm. Significant translocation of Ncs1p-GFP was not observed during conjugation/sporulation. Also, changes in glucose concentration in the media (2% and 8 %) did not seem to affect the Ncs1p level in the cells (data not shown). Cellular localization of Ncs1p-GFP along the plasma membrane is intriguing and should provide a clue in the future to help identify potential target protein(s) of Ncs1p.

Fluorescence from Ncs1p-GFP in cells grown in liquid minimum media (EMM) was initially very weak and hard to detect. However, when extracellular Ca\(^{2+}\) (>0.01 M CaCl\(_2\)) was added to EMM, the fluorescence signal from these cells increased significantly (Fig. 9B). In addition, the intracellular level of Ncs1p-GFP increased quite dramatically in cells grown in the presence of 0.01 M CaCl\(_2\) compared to that of cells grown with no added CaCl\(_2\) (Fig. 9C). These data suggest that intracellular level of Ncs1p is controlled in a highly calcium-dependent manner, consistent with its role as a Ca\(^{2+}\) sensor.
DISCUSSION

Our NMR structural analysis reveals that *S. pombe* Ncs1p is structurally similar to mammalian recoverin and exhibits a Ca\(^{2+}\)-myristoyl switch (Figs. 3 and 4). Ncs1p is 46% identical in sequence to bovine recoverin, and several conserved residues are functionally important (Fig. 1). Ncs1p binds functionally to 3 Ca\(^{2+}\) and exhibits Ca\(^{2+}\)-induced and myristoylation-dependent binding to cell membranes (Fig. 5). The amino-terminal myristoyl group is sequestered in a hydrophobic cavity of Ca\(^{2+}\)-free Ncs1p as evidenced by many NMR dipolar interactions of the myristoyl methyl group with hydrophobic side chains within the protein (Fig. 4A). The binding of three Ca\(^{2+}\) to the protein leads to the extrusion of the myristoyl group out into the solvent (no detectable protein-myristate interactions, Fig. 4B), making the attached fatty acyl group available to interact with lipid bilayers or other hydrophobic targets. The Ca\(^{2+}\)-induced exposure of the myristoyl group permits Ncs1p to associate with membrane-bound targets only at high Ca\(^{2+}\) levels.

The physiological role of the Ca\(^{2+}\)-myristoyl switch was investigated using the *ncs1Δ* mutant in fission yeast that exhibited two distinct phenotypes: starvation-independent conjugation and sporulation as well as arrested cell growth at high levels of extracellular Ca\(^{2+}\) (> 0.1 M CaCl\(_2\)). In *ncs1Δ* mutants, mammalian recoverin or *S. cerevisiae* Frq1 each complemented the starvation-independent sporulation but failed to restore tolerance at high Ca\(^{2+}\), suggesting that the two *ncs1Δ* phenotypes are mechanistically distinct. The Ca\(^{2+}\)-binding deficient triple mutant (Ncs1p\(^{E84Q/E120Q/E168Q}\)) did not influence any of the *ncs1Δ* phenotypes, demonstrating that Ca\(^{2+}\)-bound Ncs1p (and a Ca\(^{2+}\)-myristoyl switch) is physiologically active and essential in fission yeast. However, the unmyristoylated Ncs1p\(^{G2A}\) mutant partially complemented all the *ncs1Δ* phenotypes, suggesting that the N-terminal myristoyl group may not be essential for the recognition of signaling targets. Instead, the fatty acyl group may serve as a dynamic anchor to cell membranes during signaling. Consistent with this interpretation, Ca\(^{2+}\)-bound unmyristoylated recoverin is able to bind and regulate its target (rhodopsin kinase) (57) but is unable to bind to rod outer segment disc membranes (35).
We want to better understand the molecular basis and physiological relevance of Ca\(^{2+}\) intolerance in the *ncs1* deletion mutant (see Fig. 8). Interestingly, a very similar Ca\(^{2+}\) sensitivity phenotype was reported recently for the deletion mutant of an *ncs1* homolog in the plant fungus, *Magnaporthe grisea* (58) and for *prz1Δ* in fission yeast (59). The Ca\(^{2+}\) intolerance in *ncs1Δ* was not due to any defect in the osmotic stress response (60) because the intolerance was highly selective for Ca\(^{2+}\). Therefore, Ncs1p appears to protect the cell against lethal effects at elevated calcium levels. Indeed, the cellular level of Ncs1p increased markedly when cells were treated with 0.01 M CaCl\(_2\) (Fig. 9), consistent with its role as a Ca\(^{2+}\) sensor. However, the Ca\(^{2+}\) tolerance is not due to simple Ca\(^{2+}\) buffering by Ncs1p, because recoverin and/or Frq1 (both of which bind Ca\(^{2+}\) with the same capacity as Ncs1p) could not rescue the Ca\(^{2+}\) intolerance of the deletion mutant (Fig. 8B). Instead, Ca\(^{2+}\)-bound Ncs1p might specifically control Ca\(^{2+}\) influx in fission yeast by regulating ion channels. Indeed, mammalian NCS-1 negatively regulates voltage-gated Ca\(^{2+}\) channels (61), and the related K\(^+\) Channel Interacting Proteins (KChIPs) regulate the gating kinetics of A-type K\(^+\) channels (62). Alternatively, Ncs1p may serve as a calcium sensor that activates Ca\(^{2+}\)-induced expression of stress-response genes. A number of genes in *S. pombe* have been postulated to be upregulated in response to a transient increase in intracellular calcium (3,5,6,59). Furthermore, a recently discovered NCS protein called DREAM has been shown to serve as a transcriptional repressor that controls Ca\(^{2+}\)-regulated expression of c-fos and prodynorphin genes in the brain (63).

What is the molecular basis of the nutrition-insensitive sexual development in the *ncs1* deletion mutant? An important clue is that exogenously added cAMP suppresses this phenotype, suggesting that Ncs1p may regulate the cAMP pathway in sporulation turned on by the glucose sensing G protein coupled receptor, Git3p (54). Another clue is that this phenotype is complemented by the expression of retinal recoverin, which promotes Ca\(^{2+}\) regulated desensitization of the G protein coupled receptor, rhodopsin (23-25). An intriguing hypothesis is that *S. pombe* Ncs1p may regulate sporulation perhaps by controlling Ca\(^{2+}\)-dependent desensitization of Git3p, analogous to the action of Ca\(^{2+}\)-bound recoverin on rhodopsin.
Desensitization of G protein coupled receptors is generally promoted by phosphorylation of the cytosolic C-terminal tail catalyzed by cognate G protein coupled receptor kinases (GRKs) (64). A variety of Ca\textsuperscript{2+} binding proteins (such as recoverin, VILIP, NCS-1 and calmodulin) selectively regulate the activity of GRK subtypes (65,66). Therefore, Ncs1p might regulate a GRK in fission yeast that specifically phosphorylates the glucose bound form of Git3p, analogous to the action of retinal recoverin in regulating rhodopsin kinase activity (23,24). Fission yeast homologs of familiar GRKs like rhodopsin kinase or βARK have not been identified yet. However, the type I casein kinase genes (\textit{YCK1} and \textit{YCK2}) in budding yeast are known to phosphorylate the C-terminal region of the α-factor pheromone receptor (67) and therefore may serve as a specialized class of GRK genes in yeast. Indeed, fission yeast contains three homologs of \textit{YCK1} (cki1, cki2, and cki3 (68)) that might serve as cognate GRKs for \textit{git3} or pheromone receptors, \textit{mam2} and \textit{map3}. We are now in the process of identifying physiological target proteins of \textit{S. pombe} that bind to Ncs1p in a Ca\textsuperscript{2+}-regulated fashion to more rigorously understand the regulatory mechanism of conjugation and spore formation mediated by Ncs1p in fission yeast.

Another candidate target protein of Ncs1p is the fission yeast homolog of \textit{S. cerevisiae} PtdIns 4-kinase isoform, Pik1 (target of Frq1). Frq1 binds and activates Pik1, which is essential for vegetative growth of budding yeast (29). Correspondingly, \textit{S. pombe} contains a highly conserved homolog of Pik1 (SPAC22E12.16c) that most likely is activated by Ncs1p in an analogous fashion. Indeed, we see that \textit{S. pombe} Ncs1p binds tightly to residues 110-192 of \textit{S. cerevisiae} Pik1 (data not shown) like we have seen previously for Frq1 (69), suggesting that Ncs1p binds and possibly activates Pik1. Surprisingly, the \textit{ncs1Δ} mutant of fission yeast was viable and exhibited normal vegetative growth, in sharp contrast to the lethal phenotypes of \textit{frq1Δ} and \textit{pik1Δ} mutants in budding yeast (29). Therefore, if Ncs1p does activate a Pik1 homolog in fission yeast, this function is not essential for supporting vegetative growth as it is in the budding yeast. Conversely, the observation that Frq1 complements the starvation-independent sporulation phenotype of \textit{ncs1Δ} fission yeast strain suggests that Frq1 might also control nutrition regulated signaling pathways in the budding yeast.
Pheromone stimulation of budding yeast leads to a transient rise in the intracellular Ca\(^{2+}\) concentration due to the opening of stretch-activated calcium channels encoded by the \textit{MID1} gene (70,71). The fission yeast homolog of Midl (Yam8p) also has been suggested to participate in the generation of Ca\(^{2+}\) signals during pheromone stimulation of Mam2p and Map3p (5,6). It remains to be elucidated what roles, if any, Ncs1p might play in transmitting pheromone-induced Ca\(^{2+}\) signals in fission yeast.

In summary, \textit{S. pombe} Ncs1p binds functionally to intracellular Ca\(^{2+}\) and possesses a Ca\(^{2+}\)-myristoyl switch. The \textit{ncs1} gene is not essential for vegetative growth of fission yeast, in contrast to the lethal null phenotype of the budding yeast homolog, \textit{FRQ1} (29). The \textit{ncs1A} mutant exhibits nutrition insensitive sexual development and a growth defect at high levels of extracellular Ca\(^{2+}\). The starvation-independent conjugation and spore formation of \textit{ncs1} null mutants was suppressed by exogenous cAMP and by the expression of mammalian recoverin or \textit{S. cerevisiae} Frq1. We propose that Ncs1p may regulate conjugation and sporulation in fission yeast by modulating Ca\(^{2+}\)-dependent desensitization of the G protein coupled glucose receptor, Git3p.

\textbf{Acknowledgments}

We thank Dr. Henry Levin for providing \textit{S. pombe} wild type strains, plasmids pREP1, pCS2pkSu and pAF1 as well as valuable advice; Dr. David Balasundaram for initial technical support and encouragement and Dr. Harold Smith for valuable discussion; Dr. Herbert Tabor for use of dissection microscope; Dr. Jeremy Thorner for providing plasmid constructs of Frq1; and Dr. Nese Sari for help with NMR experiments.
REFERENCES

1. Catty, P., and Goffeau, A. (1996) Bioscience Reports 16, 75-85.
2. Davis, T. N. (1995) Adv. Second Messen. Phosphoprot. Res. 30, 339-358.
3. Okorokov, L. A., Silva, F. E., and Facanha, A. L. (2001) FEBS Lett. 505, 321-324.
4. Berridge, M. J. (1997) J. Physiol. 499, 291-306.
5. Carnero, E., Ribas, J. C., Garcia, B., Duran, A., and Sanchez, Y. (2000) Mol. Gen. Genet. 264, 173-183.
6. Tasaka, Y., Nakagawa, Y., Sato, C., Mino, M., Uozumi, N., Murata, N., Muto, S., and Iida, H. (2000) Biochem. Biophys. Res. Commun. 269, 265-269.
7. Facanha, A. L., Appelgren, H., Tabish, M., Okorokov, L., and Ekwall, K. (2002) J. Cell. Biol. 157, 1029-1039.
8. Halachmi, D., Ghislain, M., and Eilam, Y. (1992) Eur. J. Biochem. 207, 1003-1008.
9. Ghislain, M., Goffeau, A., Halachmi, D., and Eilam, Y. (1990) J. Biol. Chem. 265, 18400-18407.
10. Ikura, M. (1996) Trends Biochem. Sci. 21, 14-17.
11. Moser, M. J., Flory, M. R., and Davis, T. N. (1997) J. Cell. Sci. 110, 1805-1812.
12. Wu, J. Q., Bahler, J., and Pringle, J. R. (2001) Mol. Biol. Cell 12, 1061-1077.
13. Andoh, T., Kato, T., Matsui, Y., and Toh, A. (1998) Mol. Gen. Genet. 258, 139-147.
14. D'souza, V. M., Naqvi, N. I., Wang, H., and Balasubramanian, M. K. (2001) Cell Struct. Funct. 26, 555-565.
15. Baum, P., Furlong, C., and Byers, B. (1986) Proc. Natl. Acad. Sci. USA 83, 5512-5516.
16. Slupsky, C. M., Desautels, M., Huebert, T., Zhao, R., Hemmingsen, S. M., and McIntosh, L. P. (2001) J. Biol. Chem. 276, 5943-5951.
17. Burgoyne, R. D., and Weiss, J. L. (2001) Biochem. J. 353, 1-12.
18. Braunewell, K. H., and Gundelfinger, E. D. (1999) Cell Tissue Res 295, 1-12.
19. Palczewski, K., Polans, A. S., Baehr, W., and Ames, J. B. (2000) Bioessays 22, 337-350.
20. Kawasaki, H., Nakayama, S., and Kretsinger, R. H. (1998) *Biometals* **11**, 277-295.

21. Yap, K. L., Ames, J. B., Swindells, M. B., and Ikura, M. (1999) *Proteins* **37**, 499-507.

22. Dizhoor, A. M., Ray, S., Kumar, S. Niemi, G., Spencer, M., Rrolley, D., Walsh, K.A., Philipov, P.P., Hurley, J.B. and Stryer, L. (1991) *Science* **251**, 915-918.

23. Chen, C. K., Inglese, J., Lefkowitz, R. J., and Hurley, J. B. (1995) *J. Biol. Chem.* **270**, 18060-18066.

24. Kawamura, S. (1993) *Nature* **362**, 855-857.

25. Erickson, M. A., Lagnado, L., Zozulya, S., Neubert, T. A., Stryer, L., and Baylor, D. A. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 6474-6479.

26. Hidaka, H., and Okazaki, K. (1993) *Neuroscience Research* **16**, 73-77.

27. Kobayashi, M., Takamatsu, K., Saitoh, S., and Noguchi, T. (1993) *Journal of Biological Chemistry* **268**, 18898-18904.

28. Pongs, O., Lindemeier, J., Zhu, X.R., Theil, T., Engelkamp, D., Krah-Jentgens, Lambrecht, H.G., Koch, K.W., Schwemer, J., Rivosecchi, R., Mallart, A., Galeceran, J., Canal, I., Barbas, J.A., and Ferrus, A. (1993) *Neuron* **11**, 15-28.

29. Hendricks, K. B., Wang, B. Q., Schnieders, E. A., and Thorner, J. (1999) *Nature Cell Biol.* **1**, 234-241.

30. Ames, J. B., Ishima, R., Tanaka, T., Gordon, J. I., Stryer, L., and Ikura, M. (1997) *Nature* **389**, 198-202.

31. Ames, J. B., Hendricks, K. B., Strahl, T., Huttner, I. G., Hamasaki, N., and Thorner, J. (2000) *Biochemistry* **39**, 12149-12161.

32. Bourne, Y., Dannenberg, J., Pollmann, V., Marchot, P., and Pongs, O. (2001) *J. Biol. Chem.* **276**, 11949-11955.

33. Vijay-Kumar, S., and Kumar, V. D. (1999) *Nature Struct. Biol.* **6**, 80-88.

34. Tanaka, T., Ames, J. B., Harvey, T. S., Stryer, L., and Ikura, M. (1995) *Nature* **376**, 444-447.

35. Zozulya, S., and Stryer, L. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 11569-11573.

36. Dizhoor, A. M., Chen, C. K., Olshevskaya, E., Sinelnikova, V. V., Phillipov, P., and Hurley, J. B. (1993) *Science* **259**, 829-832.

37. Moreno, S., Klar, A., and Nurse, P. (1991) *Methods Enzymol* **194**, 795-823.
38. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A laboratory manual, 2 Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

39. Ray, S., Zozulya, S., Niemi, G. A., Flaherty, K. M., Brolley, D., Dizhoor, A. M., McKay, D. B., Hurley, J., and Stryer, L. (1992) Proc. Natl. Acad. Sci. USA 89, 5705-5709.

40. Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) Gene 173, 33-38.

41. Alfa, C., P. Fantes, J. Hyams, M. McLeod, and E. Warbrick. (1993) Experiments with fission yeast. Cold Spring Harbor, N.Y.

42. Duronio, R. J., Rudnick, D. A., Johnson, R. L., Linder, M. E., and Gordon, J. I. (1990) Methods 1, 253-263.

43. Muchmore, D. C., McIntosh, L. P., Russell, C. B., Anderson, D. E., and Dahlquist, F. W. (1989) Methods Enzymol. 177, 44-86.

44. Ames, J. B., Tanaka, T., Stryer, L., and Ikura, M. (1994) Biochemistry 33, 10743-10753.

45. Fisher, J. R., Sharma, Y., Iuliano, S., Piccioti, R. A., Krylov, D., Hurley, J., Roder, J., and Jeromin, A. (2000) Protein Expr. Purif. 20, 66-72.

46. Ames, J. B., Tanaka, T., Ikura, M., and Stryer, L. (1995) J. Biol. Chem. 270, 30909-30913.

47. Ames, J. B., Hamasaki, N., and Molchanova, T. (2002) Biochemistry 41, 5776-5787.

48. Paulus, H. (1969) Analyt. Biochem. 32, 91-100.

49. Flaherty, K. M., Zozulya, S., Stryer, L., and McKay, D.B. (1993) Cell 75, 709-716.

50. Ames, J. B., Porumb, T., Tanaka, T., Ikura, M., and Stryer, L. (1995) J Biol Chem 270, 4526-4533.

51. Tanaka, T., Ames, J. B., Kainosho, M., Stryer, L., and Ikura, M. (1998) J Biomol NMR 11, 135-152.

52. Lee, W., Revington, M. J., Arrowsmith, C., and Kay, L. E. (1994) FEBS Lett. 350, 87-90.

53. Panaretou, B., and Piper, P. (1996) Methods Mol. Biol. 53, 117-121.

54. Welton, R. M., and Hoffman, C. S. (2000) Genetics 156, 513-521.

55. Kawamukai, M., Ferguson, K., Wigler, M., and Young, D. (1991) Cell Regul. 2, 155-164.

56. Mochizuki, N., and Yamamoto, M. (1992) Mol. Gen. Genet. 233, 17-24.
57. Kawamura, S., Cox, J. A., and Nef, P. (1994) Biochemical and Biophysical Research Communications 203, 121-127.

58. Saitoh, K., Aire, T., Teraoka, T., Yamaguchi, I., and Kamakura, T. (2003) Biosci Biotechnol Biochem. 67, 651-653.

59. Hirayama, S., Sugiura, R., Lu, Y., Maeda, T., Kawagishi, K., Yokoyama, M., Tohda, H., Giga-Hama, Y., Shuntoh, H., and Kuno, T. (2003) J. Biol. Chem. 278, 18078-18084.

60. Toone, W. M., and Jones, N. (1998) Genes to Cells 3, 485-498.

61. Weiss, J. L., Archer, D. A., and Burgoyne, R. D. (2000) J. Biol. Chem. 275, 40082-40087.

62. An, W. F., Bowlby, M. R., Betty, M., Cao, J., Ling, H. P., Mendoza, G., Hinson, J. W., Mattsson, K. I., Strassle, B. W., Trimmer, J. S., and Rhodes, K. J. (2000) Nature 403, 553-556.

63. Carrion, A. M., Link, W. A., Ledo, F., Mellstrom, B., and Naranjo, J. R. (1999) Nature 398, 80-84.

64. Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) Annu. Rev. Biochem. 67, 653-692.

65. Sallese, M., Iacovelli, L., Cumashi, A., Capobianco, L., Cuomo, L., and De Blasi, A. (2000) Biochim. Biophys. Acta 1498, 112-121.

66. De Castro, E., Nef, S., Fiumelli, H., Lenz, S. E., Kawamura, S., and Nef, P. (1995) Biochemical and Biophysical Research Communications 216, 133-140.

67. Feng, Y., and Davis, N. G. (2000) Mol. Cell. Biol. 20, 5350-5359.

68. Kitamura, K., and Yamashita, I. (1998) Gene 214, 131-137.

69. Huttner, I. G., Strahl, T., Osawa, M., King, D. S., Ames, J. B., and Thorner, J. (2003) J. Biol. Chem. 278, 4862-4874.

70. Iida, H., Yagawa, Y., and Anraku, Y. (1990) J. Biol. Chem. 265, 13391-13399.

71. Iida, H., Nakamura, H., Ono, T., Okumura, M. S., and Anraku, Y. (1994) Mol. Cell. Biol. 14, 8259-8271.
S. pombe Ncs1p regulates sporulation and confers Ca$^{2+}$ tolerance

FOOTNOTES

1) S. pombe wild type strains 972, KGY425, KGY461, KGY553, KGY554 and SP870 were kindly provided by Dr. Henry Levin. All other strains were constructed in this study.

2) Plasmid pREP42EGFP-C is a kind gift from Dr. I.M.Hagan. Plasmid vector pREP1 is a gift from Dr. K. Maundrell, pCS2pkSu is a gift from Dr. J.R.McIntosh and pAF1 is a gift from Dr. K.L. Gould.

3) The nucleotide sequence of the ncs1 cDNA has been deposited into Genbank (AC # AY225216).

4) Three-dimensional animation movie of Ncs1p-GFP under endogenous ncs1 promoter reconstructed from optical sectioning images obtained by serial scanning (x 1000).

5) SDS polyacrylamide electrophoretic gel (SDS-PAGE) showing the final purity of recombinant myristoylated Ncs1p samples used for NMR and Ca$^{2+}$-binding studies.
**FIGURE LEGENDS**

**Figure 1.** Alignment of the primary structure of *S. pombe* Ncs1p with various members of the recoverin branch of EF-hand Ca$^{2+}$-binding proteins.

In each of the four EF-hands, the central Ca$^{2+}$-binding loop is underlined. Residues shaded black or gray and highlighted in bold are structurally important and remain invariant in Ncs1p and recoverin but are not conserved in Frq1. Swiss Protein Database accession numbers are Q09711 (*S. pombe* Ncs1p), Q06389 (*S. cerevisiae* Frq1), P21457 (bovine recoverin), P29554 (bovine neurocalcin), and P37236 (*Drosophila* frequenin).

**Figure 2.** Equilibrium Ca$^{2+}$ binding to Ncs1p.

Titrations of $^{45}$Ca$^{2+}$ binding to myristoylated Ncs1p were conducted using an ultrafiltration method, as described in Experimental Procedures. Number of ions bound per molecule of protein is plotted versus the free calcium concentration. Solid lines represent the best fit to the Hill Model using the parameters as defined in the text: $K_d = 2 \mu$M and Hill coefficient of 1.9.

**Figure 3.** Two-dimensional $^{15}$N-$^1$H HSQC NMR spectra of Ncs1p.

Two-dimensional $^{15}$N-$^1$H HSQC NMR spectra for Ca$^{2+}$-free (A) and Ca$^{2+}$-bound (B) forms of myristoylated Ncs1p, uniformly labeled with nitrogen-15, were recorded at 600-Mhz $^1$H frequency at 35 ºC.

**Figure 4.** Ca$^{2+}$-induced extrusion of the N-terminal myristoyl group.

Three-dimensional $^{13}$C(F$_1$)-edited, $^{13}$C(F$_3$)-filtered NOESY-HMQC NMR spectra of Ca$^{2+}$-free (A) and Ca$^{2+}$-bound (B) Ncs1p. The attached myristoyl group was labeled with carbon-13 and the protein was unlabeled. Selected slices at F$_2$ = 16.9 ppm are shown to specifically probe the C$_{14}$-methyl group of the fatty acyl chain.
**Figure 5.** Both Ca^{2+}-induced conformational changes and the myristoyl group contribute to membrane association of Ncs1p.

Lysates of S. pombe ncs1 deletion mutant (NHP057) overexpressing either myristoylated Ncs1p-EGFP (A) or unmyristoylated Ncs1p^{G2A}-EGFP (B) were prepared in the presence of excess Ca^{2+} (0.2 mM CaCl2) or chelator (5 mM EGTA or EDTA). The cell lysate was fractionated into insoluble and soluble fractions. Whole lysate (W), insoluble (pellet, P) and soluble (supernatant, S) fractions from the same amount of cells were analyzed by Western blot analysis. Similar experiments were carried out using ncs1Δ cells expressing GFP alone (C) and using a strain with Ncs1p-GFP coded in its genomic allele (NHP002D)(D).

**Figure 6: Deletion mutant construct.**

The restriction map of ncs1 allele of ncs1^{+} and ncs1Δ::his3 strains. S. pombe his3^{+} cassette was used as a marker in this disruption. Exons were indicated with thick lines. B: BambHI, C: ClaI.

**Figure 7: Mutants exhibit unstable diploidy and starvation-independent conjugation/sporulation.**

(A) Color test of homozygous wildtype, heterozygous or homozygous mutant diploid cells streaked on low adenine plate (YES with no adenine added). From left, NHP058(ncs1^{+}) x NHP057(ncs1Δ), NHP057(ncs1Δ) x NHP059(ncs1Δ), NHP058(ncs1^{+}) x NHP060(ncs1^{+}) and NHP060(ncs1^{+}) x NHP059(ncs1Δ). White colonies represent diploid cells and pink colonies represent haploid cells. Results of counting individual white and pink colonies are summarized below the pictures. (B) Cell morphology of wildtype h^{90} strain and its ncs1Δ mutant incubated in EMM containing 3% glucose (46 hrs) was observed microscopically. The percentage of zygotes (arrowheads) formed was 4% (ncs1^{+}) and 9% (ncs1Δ), and percentage of zygotic asci (arrows) formed was <0.4% (ncs1^{+}) and 13% (ncs1Δ). Arrowheads and arrows indicate zygotes and zygotic asci, respectively. (C) Same as in Figure 7B except carried out in the presence and absence of 10
S. pombe Ncs1p regulates sporulation and confers Ca\(^{2+}\) tolerance

mM cAMP. Picture in both Fig. 7B and 7C are taken as Nomarski images (DIC) (x 400). Black bars indicate 10 \(\mu\)m.

**Figure 8:** Deletion mutant exhibits calcium sensitivity.

(A) Growth of wild type and ncs1 mutant strains on rich media (YES) plate containing either 0.1 M CaCl\(_2\) or 0.1 M MgCl\(_2\). From bottom, clockwise, SP870 (h\(^{90}\) ncs1\(^{+}\)), NHP033 (h\(^{90}\) ncs1\(^{A}\)), NHP057 (ncs1\(^{A}\)), NHP058 (ncs1\(^{-}\)), NHP059 (ncs1\(^{A}\)) and NHP060 (ncs1\(^{-}\)). (B) Growth of ncs1\(^{A}\) (NHP057) cells overexpressing ncs1, ncs1\(^{G2A}\), ncs1\(^{3EQ}\), bovine recoverin, or S. cerevisiae FRQ1 gene on rich (YES) plate enriched with zero or 0.1 M CaCl\(_2\). From top, anticlockwise, plasmid introduced to NHP057 was pREP1-ncs1, pREP1-ncs1\(^{G2A}\), pREP1-ncs1\(^{3EQ}\), pREP1-Reco, pREP1-FRQ1 and vector pREP1.

**Figure 9:** Ncs1p is localized in cytoplasm and its cellular level is regulated by calcium.

In Figs. 9A and 9B, S. pombe strain which expresses Ncs1p-GFP under endogenous ncs1 promoter were observed by fluorescence microscopy (x 1000). Black bars indicate 10 \(\mu\)m.

(A) NHP078 (Ncs1p-GFP expressed under endogenous ncs1 promoter, h\(^{-}\) ncs1\(^{-}\)-GFP::ura4\(^{+}\)) was grown on rich plate (YES) for 48 hrs. Green fluorescent protein fluorescence (GFP), DAPI fluorescence (DAPI), and Nomarski image (DIC) are shown. When GFP alone was overexpressed, GFP signal was seen uniformly in the cytoplasm (data not shown). (B) NHP078 was grown in minimum liquid media (EMM) in the presence or absence of additional CaCl\(_2\) (0.01M or 0.1M) for 16 hrs. (C) Cell lysates of NHP078 grown in EMM containing zero or 0.01M CaCl\(_2\) were analyzed by Western blot analysis (left panel). Right panel shows equivalent gel stained by CBB to show that each lane contained the same level of protein.
Table 1

*S. pombe* strains.

| Strain  | Genotype                                      |
|---------|-----------------------------------------------|
| 972     | \( h^- \) canonical wild type                |
| KGY425  | \( h^- \) his3-D1 ade6-M210 leu1-32 ura4-D18 |
| KGY461  | \( h^+ \) his3-D1 ade6-M210 leu1-32 ura4-D18 |
| KGY553  | \( h^- \) his3-D1 ade6-M216 leu1-32 ura4-D18 |
| KGY554  | \( h^+ \) his3-D1 ade6-M216 leu1-32 ura4-D18 |
| SP870   | \( h^{90} \) ade6-M210 leu1-32 ura4-D18      |
| NHP033  | \( h^{90} \) ade6-M210 leu1-32 ura4-D1 ncs1\(\Delta\)::ura4 |
| NHP002  | \( h^- \) his3-D1 ade6-M210 leu1-32 ura4-D18 ncs1\(^+\)-GFP::ura4\(^+\) |
| NHP078  | \( h^+ \) his3-D1 ade6-M210 leu1-32 ura4-D18 ncs1\(^+\)-GFP::ura4\(^+\) |
| NHP001D | \( h^+/ h^- \) his3-D1/ his3-D1 ade6-M210/ ade6-M216 leu1-32/ leu1-32 ura4-D18/ ura4-D18 |
| NHP009D | \( h^+/ h^- \) his3-D1/ his3-D1 ade6-M210/ ade6-M216 leu1-32/ leu1-32 ura4-D18/ ura4-D18 ncs1\(^+\)/ncs1\(\Delta\)::his3 |
| NHP013  | \( h^- \) his3-D1 ade6-M216 leu1-32 ura4-D18 ncs1\(\Delta\)::his3 |
| NHP057  | \( h^- \) his3-D1 ade6-M210 leu1-32 ura4-D18 ncs1\(\Delta\)::his3 |
| NHP058  | \( h^+ \) his3-D1 ade6-M216 leu1-32 ura4-D18 |
| NHP059  | \( h^+ \) his3-D1 ade6-M216 leu1-32 ura4-D18 ncs1\(\Delta\)::his3 |
| NHP060  | \( h^- \) his3-D1 ade6-M210 leu1-32 ura4-D18 |
Table 2: $^{45}\text{Ca}^{2+}$ binding data obtained from ultrafiltration assay.

| Total Ca$^{2+}$ (µM) | $R_{\text{filtrate}}/R_{\text{sample}}$ | Free Ca$^{2+}$ (µM) | Bound Ca$^{2+}$/Protein |
|----------------------|----------------------------------------|----------------------|-------------------------|
| 5.5                  | 0.0454                                 | 0.25                 | 0.075                   |
| 15.7                 | 0.0412                                 | 0.65                 | 0.225                   |
| 28.7                 | 0.0313                                 | 0.90                 | 0.41                    |
| 52.5                 | 0.0228                                 | 1.2                  | 0.75                    |
| 78.7                 | 0.0216                                 | 1.7                  | 1.12                    |
| 131                  | 0.0191                                 | 2.5                  | 1.87                    |
| 180                  | 0.0444                                 | 8.0                  | 2.57                    |
| 240                  | 0.1667                                 | 40                   | 2.85                    |
| 300                  | 0.3160                                 | 95                   | 2.93                    |
| 400                  | 0.4592                                 | 184                  | 3.09                    |
| 500                  | 0.5646                                 | 282                  | 3.11                    |
| 600                  | 0.6581                                 | 395                  | 2.93                    |

$R_{\text{filtrate}}/R_{\text{sample}}$ is equivalent to $[\text{Ca}^{2+}_{\text{free}}]/[\text{Ca}^{2+}_{\text{tot}}]$ and was determined as the ratio of $^{45}\text{Ca}^{2+}$ radioactivity measured from equal aliquots of filtrate and sample compartments during the ultrafiltration Ca$^{2+}$ binding assay as described in Experimental Procedures. Free Ca$^{2+}$ concentration is calculated as the total Ca$^{2+}$ concentration times $R_{\text{filtrate}}/R_{\text{sample}}$. Bound Ca$^{2+}$ concentration is calculated as the total Ca$^{2+}$ minus free Ca$^{2+}$ (protein concentration was 70 µM).
**Table 3:** Percentage of spore formation in nutrient-rich medium using homothallic *ncs1Δ* strain (NHP033) bearing expression plasmid with corresponding gene.

| Gene     | Zygotic asci (%) |
|----------|------------------|
|          | Exp. 1 Liquid, 24 hrs. | Exp. 2 Liquid, 46 hrs. | Exp. 3 Plate, 6 days |
| Vector   | 16.7             | 24.2             | 23.8             |
| *ncs1*   | 3.0              | 13.1             | 14.8             |
| *ncs1*<sup>G2A</sup> | 5.7 | 6.6 | 12.0 |
| *ncs1*<sup>3EQ</sup> | 11.7 | 21.7 | 23.5 |
| *Reco*   | 4.7              | 7.4              | 9.5              |
| *FRQ1*   | 2.0              | 8.3              | 4.2              |
**Figure 1**

| Protein          | Sequence |
|------------------|----------|
| Ncs1p         | MGHSQK.LS QDQLQDLVRS TRFDKQELQQ WYKGFRDPCP SGHLNKSEEFQ |
| Frq1             | MGAKTSK.LS KDDLTCLKQS TYPDREEIQQ WHKGLRDPCP SGQLAREDFV |
| Recoverin       | MGNSKG GALS KEILEELQLN TKFTEEELS YWQSLKRPDCP SGRITRQEPF |
| Frequenin       | MGKKSSK.LK QT丁DTLRKTD YTFKERIQQ WHKGFLKDPCP NGLLTEQGF |
| Neurocalcin     | MGKQNSK.LR PEVMQDLLES TDFTHEIQE WYKGFLRDPCP SGHLSMEFK |

| Protein          | Sequence |
|------------------|----------|
| Ncs1p         | KIYKQFFPFG DPSAFAEYVF NVFDADKNGY IDEKREFICAL SVTSRGELND |
| Frq1             | KIYKQFFPFG SPEDFANHFL TVFDKDNNGF IHEEFITVL STTSRGTLEQ |
| Recoverin       | TIYSKFPPEA DPRQYAHQVF RSPDNSDGFTI DKEKYVIAL HMTSAGKTNQ |
| Frequenin       | KIYKQFFPQG DPSKASLVF RVDENNDGS IDEEFIRAL SVTSKGNLDE |
| Neurocalcin     | KIYGNFFPYG DASKFAEYVF RTFDAKDGT IDEFIIAL SVTSRKLLEQ |

| Protein          | Sequence |
|------------------|----------|
| Ncs1p         | KLWAFQLYD IDENGSLSYD EMLRIVDAIY KMVG..SMVKEPEDEDTPFK |
| Frq1             | KLSWAFELYD LINNGYITFD EMLTTASVIY KMMG..SMVITEDEATPEK |
| Recoverin       | KLEWAFLSLID VDGNTISQN EVLEIVMAIF KMISPEDTKH IPEDENTPEK |
| Frequenin       | KLQWAFRLYD VDENGITRE EMNIVDAIY QMVG..QQPQ .SEDESTPQK |
| Neurocalcin     | KLMWAFSMYD LDENGYISKA EMLEIVQAIY KMVG..SVMK MEPEDESTPEK |

| Protein          | Sequence |
|------------------|----------|
| Ncs1p         | RVNKIFNMMD KNKDGQLELE EFCEGSKRDP TIVSALSLYD GLV |
| Frq1             | RKKIFKLMND KNEDGYTLD EFREGSKVDP SIIANLNYD GLI |
| Recoverin       | RAEEKWFGFF KGKDDKLLER EFIEGTTLANK EIILQIFEF PQVEKELKEK KL |
| Frequenin       | RVDFKIPQMD KHNIDGKITLE EFREGSKADP RIVQALSLLG G |
| Neurocalcin     | RTEKIFRQMD TNRDGKLSEL EFIRGAKSDP SIVRLLQCDP SSAGQF |
Figure 2

S. pombe Ncs1p regulates sporulation and confers Ca\(^{2+}\) tolerance

Calcium (M)

Bound Ca\(^{2+}\)/Protein
Figure 3
**Figure 4**

A. Ca\(^{2+}\)-free

\(^{13}\)C (F\(_2\)): 16.8 ppm

B. Ca\(^{2+}\)-bound

\(^{13}\)C (F\(_2\)): 16.9 ppm
Figure 5
Figure 6
A

Color test

|                | Exp. 1 | Exp. 2 |
|----------------|--------|--------|
| **White colonies (%) ±SD** |        |        |
| *ncs1*+ x *ncs1Δ*     | 9 ±1   | 14 ±4  |
| *ncs1Δ* x *ncs1Δ*     | 11 ±3  | 14 ±4  |
| *ncs1*+ x *ncs1*+     | 54 ±15 | 57 ±20 |
| *ncs1Δ* x *ncs1*+     | 8 ±1   | 10 ±1  |

Figure 7A
Figure 7B
Figure 7C

C

(ncs1+) -cAMP

(ncs1Δ) -cAMP

(ncs1+) +cAMP

(ncs1Δ) +cAMP
Figure 8
Figure 9A
Figure 9B
\textbf{S. pombe} Ncs1p regulates sporulation and confers Ca\textsuperscript{2+} tolerance

\medskip

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Western Blot} & \textbf{CBB Staining} & \textbf{CaCl}_2 added & \textbf{CaCl}_2 added \\
\hline
\text{None} & \text{None} & \text{0.01M} & \text{0.01M} \\
\hline
\end{tabular}
\caption{Western Blot and CBB Staining with/without CaCl\textsubscript{2} addition.}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{Figure_9C}
\caption{Figure 9C}
\end{figure}
Fig. S1: Purification profile of myristoylated Ncs1p expressed in *E. coli*. Lanes of SDS-PAGE gel: Molecular weight standards (1), *E. coli* lysate (2), soluble extract (3), Phenyl-sepharose flow-through fraction (4), Phenyl-sepharose elution (5), DEAE-sepharose elution (6), Sephacryl-S100 purified sample used for NMR (7), and purified bovine recoverin used in previous NMR experiments (8).
Fission yeast homolog of neuronal calcium sensor-1 (Ncs1p) regulates sporulation and confers calcium tolerance

Nobuko Hamasaki-Katagiri, Tatiana Molchanova, Kazuyo Takeda and James B. Ames

*J. Biol. Chem.* published online January 13, 2004

Access the most updated version of this article at doi: [10.1074/jbc.M311895200](http://doi.org/10.1074/jbc.M311895200)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
[http://www.jbc.org/content/suppl/2004/04/19/M311895200.DC1](http://www.jbc.org/content/suppl/2004/04/19/M311895200.DC1)