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Identification and characterization of Csh3 as a SH3 protein that interacts with fission yeast Cap1

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Abstract

Schizosaccharomyces pombe Cap1 has been identified as the (adenylyl) cyclase associated protein. Cap1 was able to form homomers , and also binds actin. Cap1 localized to the growing tip, and this localization was dependent on the the P2 region in the middle domain. In a two-hybrid screening using *cap1* as a bait, we isolated *csh3*, which encodes a protein of 296 amino acids with an SH3 domain and a proline/glutamine rich region. The binding of Csh3 and Cap1 was confirmed by *in vivo*

pull down assays. Cooperative functions of Csh3 and Cap1 were observed. Deletion of both *csh3* and *cap1* resulted in heightened sensitivity to $CaCl_2$, while disruption of either gene alone did not have any effect in this regard. In addition, over-expression of *csh3* or *cap1* alone did not affect cell growth, while over-expression of both genes resulted in growth retardation. Finally, while Csh3-GFP localized to the cytoplasm in wild-type cells, its localization was altered in *cap1*Δ cells, suggesting that the interaction between Csh3 and Cap1 controls the cellular localization of Csh3. These results demonstrate that Cap1 in *S. pombe* is a multifunctional protein that functions through dimerization (or multimerization) and interaction with other proteins including adenylyl cyclase, actin and a protein Csh3.

Introduction

The adenylyl cyclase-associated protein, CAP, and its homologues have been identified in all eukaryotes studied including yeasts, fungi, fly, plants, and mammals (Field, *et al.*, 1990, Kawamukai, *et al.*, 1992, Matviw, *et al.*, 1992, Marcus, *et al.*, 1993, Vojtek & Cooper, 1993, Gottwald, *et al.*, 1996, Kawai, *et al.*, 1998, Zhou, *et al.*, 1998, Bahn & Sundstrom, 2001, Zhou, *et al.*, 2012). CAP was first identified as a component of the adenylyl cyclase complex in the budding yeast *Saccharomyces cerevisiae* (Field, *et al.*, 1990), and later in the fission yeast *Schizosaccharomyces pombe* (Kawamukai, *et al.*, 1992). In both yeasts, CAPs were identified as adenylyl cyclase-associated proteins, and its association with adenylyl cyclase has been shown in some other fungi (Bahn & Sundstrom, 2001, Zhou, *et al.*, 2012), but in higher eukaryotes, the designation as a CAP protein was based solely on the structural similarity and the conservation of the C-terminal domain functions with yeast CAPs (Matviw, *et al.*, 1992, Marcus, *et al.*, 1993, Vojtek & Cooper, 1993, Kawamukai, 1996, Kawai, *et al.*, 1998). The C-terminal domain of all CAPs possesses a well-conserved actin binding function (Hubberstey & Mottillo, 2002, Jansen, *et al.*, 2014). The phenotype associated with C-terminal deletion of yeast CAP can be rescued by expression of the C-terminal domain of mammalian CAP homologs, suggesting that the C-terminal domain has functional similarity across multiple species (Matviw, *et al.*, 1992, Vojtek & Cooper, 1993). CAP mutants displaying defects in the actin cytoskeleton have been described not only in yeasts (Vojtek & Cooper, 1993), but also in *Dictyostelium* (Gottwald, *et al.*, 1996), *Drosophila* (Baum, *et al.*, 2000) and mammals (Zhang, *et al.*, 2013). The C-terminal domain of CAP has been shown to displace cofilin from ADP-G-actin and catalyzes monomer nucleotide exchange (Mattila, *et al.*, 2004).

CAP has been most thoroughly studied in *S. cerevisiae*, in which it has been found to be a multi-functional protein (Gerst, *et al.*, 1991, Freeman, *et al.*, 1995, Freeman, *et al.*, 1996). The N-terminal region of CAP interacts with adenylyl cyclase and this interaction is required for the activation of the adenylyl cyclase by RAS (Nishida, *et al.*, 1998, Shima, *et al.*, 2000). It has been suggested that the N-terminal 36 residues of CAP are sufficient and specifically important for the activation of adenylyl cyclase by modified RAS (Nishida, *et al.*, 1998, Shima, *et al.*, 2000), while the C-terminal region has a role in maintaining the actin cytoskeleton (Gerst, *et al.*, 1991, Freeman, *et al.*, 1995, Zelicof, *et al.*, 1996). The functions of the N-terminal and the C-terminal domains seemed to be independent of each other. However, actin organization is linked to the activation of the Ras signaling pathway through CAP (Gourlay & Ayscough, 2006) , which suggests a

functional link between these two seemingly independent domains of CAP. Recent studies indicated that while the C-terminus of CAP has a role of binding and recycling actin monomers, the N-terminus of CAP has cofilin-mediated severing activity of actin filaments. Between the N-terminus and the C-terminus, CAP contains WH2 (Wasp homology 2) domain flanked by two polyproline regions referred to as P1 or P2. WH2 plays an important role in recharging actin monomers to facilitate actin turnover. Although the P1 polyproline region is highly conserved between CAP homologues, its function remains unknown. P2 contains a consensus SH3 (*Src* Homology 3)-binding motif (PXXP) and is involved in the localization of CAP to actin patches. SH3 domains are composed of about 50 amino acids that mediate protein-protein interactions predominantly with proline rich regions. In *S. cerevisiae,* the SH3 protein Abp1p, which contains an SH3 domain, interacts with P2 and is required for the localization of CAP (Freeman*, et al.*, 1996, Hubberstey*, et al.*, 1996, Lila & Drubin, 1997).

In both yeast and mammals, CAPs are involved in recycling G-actin monomers from ADF/cofilins for subsequent rounds of filament assembly (Moriyama & Yahara, 2002). All CAPs contain a C-terminal actin-binding domain that regulates actin remodeling in response to cellular signals and is required for normal cellular morphology, cell division, growth and locomotion in eukaryotes (Hubberstey & Mottillo, 2002). The proline-rich domain of CAP interacts with profilin, a protein that catalyzes nucleotide exchange on G-actin monomers and promotes addition to barbed ends of filamentous F-actin (Bertling*, et al.*, 2007). Three-dimensional structures of CAP N- and C-terminals have been independently resolved. The N-terminal domain of CAP proteins has an all-alpha helix structure consisting of six helices in a bundle with a left-handed twist and an up-and-down topology (Ksiazek*, et al.*, 2003) and forms hexameric shurikens (Chaudhry*, et al.*, 2013). The C-terminal domain of CAP proteins has a superhelical structure, where the superhelix turns are made of two beta-strands each (Dodatko*, et al.*, 2004).

The *cap1* gene of the fission yeast *S. pombe* encodes the second Cap to be formally identified as an adenylyl cyclase-associated protein. Disruption of the entire gene resulted in defective growth in minimal medium, temperature sensitivity at 37 ˚C, and abnormal morphology (Kawamukai*, et al.*, 1992). The broad phenotype of the *cap1* disruptant suggested multiple roles for CAP, but the underlying molecular mechanisms have not been elucidated. The adenylyl cyclases in *S. cerevisiae* and *S. pombe* are regulated by different G-proteins, namely Gα in *S. pombe* and the RAS oncoprotein homolog in *S. cerevisiae* (Yamamoto, 2003). The extent of functional overlap between

the CAP proteins of *S. cerevisiae* and *S. pombe* has not been thoroughly investigated. In this study, we examined the role of Cap1 in fission yeast by looking for the Cap1-interacting protein(s), in addition to actin binding and Cap1 homomerization, and we have identified a protein named Csh3, which contains a SH3 domain and functions cooperatively with Cap1.

Materials and Methods

Yeast strains and media

The genotypes of all yeast strains used in this study are listed in Table 1. *S. pombe* strains were grown in YEA medium (0.5% yeast extract, 3% glucose and 0.0075% adenine) or PM synthetic medium containing nutritional supplements when necessary (Alfa*, et al.*, 1993). *S. cerevisiae* strains were grown in YPD medium (2% peptone, 1% yeast extract and 2% glucose) or SC synthetic medium (0.67% yeast nitrogen base, 2% glucose) with appropriate auxotrophic supplements. The yeasts were transformed using either lithium acetate (Okazaki*, et al.*, 1990) or electroporation (Prentice, 1992). General genetic methods used for *S. pombe* have been described previously (Moreno*, et al.*, 1991). The thiamine-repressible *nmt1* promoter was repressed by adding 5 μg/ml thiamine to PM medium.

DNA manipulations

Cloning, restriction enzyme analysis and preparation of plasmid DNAs were performed essentially as described (Sambrook*, et al.*, 1989). *Escherichia coli* strain DH5α was used for construction and propagation of plasmids. DNA sequences were determined by the dideoxynucleotide chain-termination method using the ABI377 DNA sequencer.

Plasmids

S1

Yeast two-hybrid screen

Strain L40 carrying pBTM116-cap was transformed with an *S. pombe* cDNA library constructed in pGAD GH (Clontech). Transformants were screened for growth on SC-Leu-Trp-His synthetic medium. His+ colonies were then placed on a filter and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for β-galactosidase activity. The plasmids derived from the library were recovered from the His+ and lacZ+ positive clones obtained through this screening, followed by the transformation of *E.*

coli. The recovered plasmids were transformed again into L40 carrying pBTM116-cap to select clones that reproducibly conferred the lacZ+ phenotype. The nucleotide sequences of the DNA inserts in the clones of interest were determined using ABI PRISMTM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer).

Disruption of the *csh3* gene

The *csh3* disruptant (*csh3*Δ) was generated as follows. A haploid strain SP870 and a diploid strain SP826 were transformed with the *csh3::ura4* fragment from pBS-csh3::ura4. Stable Ura+ transformants were selected and analyzed by Southern blot analysis to verify the proper replacement of the chromosomal *csh3* alleles by the disrupted allele. The *csh3* and *cap1* double disruptant (*csh3*Δ *cap1*Δ) was generated as follows. In the *csh3* disruptant, the *ura4* gene that disrupted the *csh3* gene was replaced with a *ura4::ADE2* fragment. The *ura4::ADE2* fragment was amplified from the *shk1* mutant by PCR (Marcus*, et al.*, 1995). Stable Ade+ transformants were selected and analyzed by Southern blot analysis. This strain was crossed with *cap1* disruptant MK1818d. Stable Ura+ Ade+ transformants were selected and analyzed by Southern blot analysis.

In vivo co-precipitation assays

Cells were grown in the absence of thiamine to an optical density at 600 nm of ~0.5 and harvested by centrifugation, followed by washing with stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, and 1 mM NaN_3 (pH 8.0)). Cells were suspended in lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet-P40, 5 mM EDTA, 10% glycerol, 1 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) and ground by glass beads (vortexing for 4 x 15 seconds), and supernatant of the cell extract was prepared by centrifugation (2,500 rpm for 10 min at 4 ˚C). Cell extract (1 μg in lysis buffer) was incubated with 30 μl of Glutathione-SepharoseTM 4B beads (Pharmacia) for 1 hour at 4 ˚C. Beads were washed five times with 0.5 ml of PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, and 1.47 mM KH_2PO_4), and re-suspended in loading buffer. After boiling for 5 min, samples were separated by SDS-PAGE using 10% polyacrylamide gels, and transferred to ImmobilonTM transfer membrane (Millipore). Immunoblotting was done with anti-GFP polyclonal antibody (Clontech) and anti-GST polyclonal antibody as primary antibodies, and with HRP-conjugated goat anti-rabbit IgG (Bio-Rad) as a secondary antibody, together with ECL reagent (Amersham).

GFP fluorescence microscopy

GFP was visualized with illumination at 485 nm using a Carl Zeiss Axioskop microscope equipped with a Hamamatsu C5985 charged-coupled device (CCD) camera. Images were taken at a magnification of 1,000x.

Results

***S. pombe* Cap1 dimerizes (or multimerization) and interacts with actin**

It was previously reported that CAP dimerizes and interacts with actin in *S. cerevisiae* and mammals (Freeman*, et al.*, 1995, Hubberstey*, et al.*, 1996, Yu*, et al.*, 1999). To determine whether these interactions are conserved in *S. pombe*, co-precipitation experiments were performed. First, to determine whether Cap1 dimerizes, *S. pombe* wild-type strain was co-transformed with two plasmids that expressed GST-fused Cap1 (Cap1-GST) and GFP-fused Cap1 (Cap1-GFP). As negative controls, plasmids expressing GST or GFP alone were used. GST fused proteins were pulled down by glutathione-Sepharose beads, and GFP-fused proteins in precipitates were probed with an anti-GFP polyclonal antibody. As shown in Fig. 1A, Cap1-GST, but not GST alone, co-precipitated with Cap1-GFP. These data suggest that *S. pombe* Cap1 forms homodimers (or multimers). To test whether Cap1 interacts with actin, GST-fused proteins were pulled down using glutathione-Sepharose beads, and the presence of actin in the precipitates was determined using an anti-actin polyclonal antibody. As shown in Fig. 1B, Cap1-GST, but not GST alone, co-precipitated with actin. Thus, *S. pombe* Cap1 dimerizes (or multimerizes) and associates with actin *in vivo*. The interaction of human CAP1 with fission yeast actin was also observed (data not shown), further supporting the conservation of Cap1 as an actin binding protein. We also sought to identify the region of Cap1 that is involved in actin binding, using the yeast two-hybrid system and several *cap1* deletion mutants. Using this system, we observed that the full-length and the C-terminal (residues 401-551) Cap1 proteins interacted with actin, but not other Cap1 fragments containing only the N-terminus or central regions (Fig. 1C).

In *S. cerevisiae*, dimerization of CAP is mediated through the C-terminal region (Zelicof*, et al.*, 1996). In mammals, however, both the N-terminal and the C-terminal regions of CAP can interact with each other or with themselves. To determine which regions of *S. pombe* Cap1 are involved in Cap1 dimerization, the yeast two-hybrid test was performed. Cap1-FL (full-length), Cap1-NT (residues 1-296), and Cap1-CT (residues 401-551) were fused to the GAL4 DNA-binding domain or the GAL4 activator domain. The

results showed that both the N-terminal and the C-terminal domains of *S. pombe* Cap1 interacted with themselves, but not with each other (Fig. 2A). To determine more precisely the regions that are involved in the dimerization of Cap1 via the N-terminal domain, various fragments were fused to the GAL4 activator domain, and these constructs were tested for interaction with Cap1 or Cap1 N.T. fused to the GAL4 DNA-binding domain (Fig. 2B). $Cap1^{1-196}$ interacted with the N-terminus of Cap1, but no binding was observed with $Cap1^{1-69}$, $Cap1^{167-236}$, and $Cap1^{167-296}$. These results indicate that the residues from 70 to 167 amino acids are important for the Cap1 dimerization (Fig. 2B).

Identification of Csh3 as a Cap1-interacting protein To identify novel proteins that can interact with *S. pombe* Cap1, yeast two-hybrid screening was performed using *cap1* as bait together with a *S. pombe* cDNA library constructed in pGAD GH (Clontech). Several positive plasmids were obtained and the inserts sequenced. This approach identified actin, cofilin, elongation factor 1α and ribosomal protein L5 as Cap1-interacting proteins. Because actin and cofilin are known to interact with Cap1 as shown above, the identification of actin and cofilin as binding partners provided an excellent internal validation of the two-hybrid screening protocol. In addition to these binding partners, we identified one positive plasmid, designated pGAD-SH3, which contained sequences coding for a protein uncharacterized at the time. Because this uncharacterized protein possessed a typical SH3 domain and also interacted with Cap1, we designated this protein as Csh3 (Cap1-interacting SH3 protein) and investigated it further.

The first clone that we isolated contained only a partial cDNA sequence (corresponding to 121 aa - 296 aa), and we sought to obtain the full-length cDNA of *csh3*. We screened a cDNA library constructed in λZAPII by plaque hybridization using the 330bp *Eco*RI - *Hae*II digested fragment of pGAD-SH3 as a probe. Two positive clones were obtained after screening about 2×10^5 plaques. Plasmids were recovered from λZAPII clones by the *in vivo* excision procedure. Sequence analysis showed that both plasmids (designated pBS-csh3-1) contained the full-length *csh3* gene, because we found in-frame stop codons located at 24 bps upstream from the 5' start codon as well as at the 3' end of the coding sequence. The cDNA sequence of *csh3* is deposited in GenBank/EMBL/DDBJ under accession number AB011825. The *csh3* gene contains no intron according to the *S. pombe* genome database (Sanger Centre). It encodes a protein of 296 amino acids that contains an SH3 domain in the center and a C-terminal region enriched in proline and

glutamic acid (designated P/Q domain). Csh3 shares homology with YGR136w (Lsb1) or YPR154w (Lsb2) (Fig. S1), both of which can bind Las17, a homolog of human Wiskott-Aldrich syndrome protein that is involved in the actin assembly in *S. cerevisiae* (Madania*, et al.*, 1999).

Csh3 interacts with the N-terminus of Cap1.

We sought to determine if the full-length Csh3 protein also interacts with Cap1, since the original two-hybrid results were obtained with only a partial *csh3* sequence. A positive signal in the two-hybrid test was obtained, using Csh3 (full-length) fused to the LexA DNA-binding domain and Cap1 fused to the GAL4 activator domain (data not shown). To confirm the results of the two-hybrid interaction between Csh3 and Cap1, co-precipitation experiments were performed. The *S. pombe* wild-type strain was co-transformed with a plasmid expressing Cap1-GST and a plasmid expressing Csh3-GFP. GST-fused Cap1 was pulled down using glutathione-Sepharose beads, and an anti-GFP polyclonal antibody was used to detect the presence of GFP-fused Csh3 in the precipitate. Cap1-GST, but not GST alone, co-precipitated with Csh3-GFP (data not shown), thus confirming the interaction between Csh3 and Cap1. We also tested the domain(s) of Cap1 that are important for its interaction with Csh3. For these studies, a *cap1*Δ *S. pombe* strain was used to avoid the possibility of false positives arising from the formation of dimers between endogenous Cap1 and exogenous Cap1 deletion mutants. As shown in Fig. 4, Cap1-GST, $Cap1^{1-236}$-GST, Cap1ΔP1-GST, and Cap1ΔP2-GST all interacted with Csh3, while deletion of both proline-rich regions (Cap1ΔP12-GST) was associated with reduced interaction with Csh3. GST alone and $Cap1^{238-551}$-GST did not co-precipitate with Csh3. Taken together, these results indicate that Csh3 is capable of interacting with Cap1 *in vivo*, and that this interaction is mediated through the N-terminus (residue 1-236) and proline rich regions of Cap1. The expression level of Csh3 did not differ substantially among various Cap1 mutant transformants (Fig. 4C). It is interesting to note that when we tested the same Cap1/Csh3 interactions in a wild-type *S. pombe* strain, we observed an interaction in all cases. This supports our original speculation that false positives could result from a 3-way interaction between endogenous (wild-type) Cap1-transfected Cap1 mutants and Csh3.

Csh3 forms dimers (or multimers)

We compared the properties of Csh3 and Cap1 by testing whether Csh3 is also able to dimerize and whether it interacts with actin. Using the yeast two-hybrid system, we

found that Csh3 does dimerize (Fig. 4), but is unable to interact with actin (data not shown). To determine the region of Csh3 that is essential for dimerization, Csh3 (full-length), Csh3ΔNT (lacking the N-terminal region, residues 1-120), or Csh3ΔSH3 (lacking the central region, residues 126-213, including the SH3 domain) were fused to the GAL4 DNA-binding domain or the GAL4 activator domain. The two-hybrid test using those constructs indicated that the formation of dimers is dependent on the N-terminal region of Csh3 (Fig. 4A). To confirm the results from the two-hybrid test, co-precipitation experiments using *S. pombe* lysate were performed. *S. pombe* wild-type strain was co-transformed with plasmids expressing Csh3-GST and Csh3-GFP. GST-fused proteins were precipitated using glutathione-Sepharose beads, and GFP-fused proteins in the precipitates were detected using an anti-GFP polyclonal antibody. Interaction between Csh3-GST and Csh3-GFP was detected, while no binding was observed between Csh3-GST and GFP alone (Fig. 4B). We also tested the interaction of Csh3 with *S. cerevisiae* CAP and human Cap1 in a two-hybrid system, but no positive results were observed (data not shown).

Genetic and functional interactions between Csh3 and Cap1

We next investigated the function of *csh3* by genetic deletion and over-expression. Plasmid-directed expression of *csh3* and *cap1* was driven by the *adh1* promoter. No significant effect on cell morphology and growth was observed when *csh3* was over-expressed alone in wild-type *S. pombe*. However, when both *csh3* and *cap1* were over-expressed, we observed growth inhibition (Fig. 5A), while normal cell morphology was maintained.

To disrupt the *csh3* gene, the *csh3::ura4* DNA fragment was generated by inserting the *ura4* gene cassette in the *Hin*d III site of *csh3* on the plasmid. The *csh3::ura4* fragment was used to transform both a haploid strain SP870 and a diploid strain SP826. Stable Ura+ colonies were isolated from both strains, and replacement of *csh3* by *csh3::ura4* was confirmed by Southern blot analysis (data not shown). Cellular proliferation and morphology were unaffected by *csh3* gene deletion. Similarly, *csh3* deficiency did not affect cellular responses to a variety of stresses such as salts (KCl, NaCl and $CaCl_2$) and temperature (25.0-36.5˚C) (data not shown). We then generated a *csh3* and *cap1* double disruptant (*csh3*Δ *cap1*Δ) and analyzed its phenotype. Growth on rich media was unaffected by *cap1* and/or *csh3* deficiency. However, the *csh3*Δ *cap1*Δ strain displayed a much severer growth defect on minimal medium than the strains with a single disruption (*csh3*Δ or *cap1*Δ). In addition, the *csh3*Δ *cap1*Δ strain showed marked growth inhibition

following exposure to 0.1 M $CaCl_2$ (Fig. 5B). Taken together, these results suggest functional cooperation between Cap1 and Csh3.

Subcellular localization of Csh3 and Cap1

To determine subcellular localization of Csh3 and Cap1 in *S. pombe*, *csh3* and *cap1* were fused with the gene encoding GFP under the control of the *nmt1* promoter. In the absence or the presence of thiamine, both GFP-fusion proteins were able to rescue the $CaCl_2$ sensitivity of *csh3*Δ *cap1*Δ strain, indicating that the GFP-fusion proteins were functional. Following transformation, Csh3-GFP localized to the cytoplasm, while Cap1-GFP localized to cortical patches at the growing ends or septum (Fig. 6). Because Csh3 and Cap1 interact with each other *in vivo*, we hypothesized that the localization of Csh3 and CAP may be affected by each other. To test this, the *csh3*Δ and *cap1*Δ strain were transformed with Cap1-GFP and Csh3-GFP, respectively. Cap1-GFP expressed in the *csh3*Δ cells had a similar localization to that in the wild-type cells, suggesting that Csh3 does not influence the localization of Cap1. However, Csh3-GFP in *cap1*Δ cells localized mainly to the cell surface, suggesting that Cap1 influences the localization of Csh3 (Fig. 6).

In *S. cerevisiae,* the localization of CAP is regulated by its proline-rich region. To determine whether this is also the case with Cap1 in *S. pombe*, *cap1*Δ strain was transformed with a plasmid that expressed Cap1ΔP1-GFP, Cap1ΔP2-GFP, Cap1ΔP12-GFP, or $Cap1^{238\text{-}551}$-GFP and cells were observed under a fluorescent microscope. As shown in Fig. 6, Cap1ΔP1-GFP localized to cortical patches at the growing ends or septum, while Cap1ΔP2-GFP mainly localized to the cytoplasm. Thus, the P2 region is important for the localization of Cap1.

Actin cytoskeletal alterations in strains with *csh3 and*, *cap1 deletions*.

Because Cap1 interacts with actin in *S. pomb*e, we examined the actin structure by staining actin filaments with rhodamine-conjugated phalloidin in *csh3*Δ, *cap1*Δ, and *csh3*Δ *cap1*Δ strains. While both the actin patches and the actin rings were observed in wild-type and *csh3*Δ strains, actin patches were not properly formed in *cap1*Δ and *csh3*Δ *cap1*Δ strains (Fig. 7). The actin localization to the growing tips was disturbed in these latter two strains, consistent with the function of Cap1 as an actin-binding protein. However, we did not observe differences in actin filament structure between *cap1*Δ and *csh3*Δ *cap1*Δ strains, suggesting that Csh3 is probably not important for the actin dynamics.

Discussion

In this study, we have identified the novel Cap1-interacting protein in *S. pombe* named Csh3 that has an SH3 domain and a P/Q rich region. Cap1 from *S. pombe* was previously shown to interact with adenylyl cyclase (Kawamukai*, et al.*, 1992) and 14-3-3 (Zhou*, et al.*, 2000), and the current study extends these findings by identifying actin and Csh3 and additional Cap1-binding proteins (Fig. 8). Binding of all four proteins with Cap1 has been verified in both the yeast two-hybrid test and GST-pull down experiments, although binding of 14-3-3 with Cap1 only occurred when the N-terminus portion, and not the full-length, of Cap1 was used (Zhou*, et al.*, 2000). The genes for ribosomal protein L5 and EF1-alpha were repeatedly isolated in our yeast two-hybrid studies, and they may be additional binding proteins of Cap1. This conclusion is supported by the previous identification of ribosomal protein L3 and EF1-alpha as CAP-binding proteins in *S. cerevisiae* (Yanagihara*, et al.*, 1997). Thus, in both *S. pombe* and *S. cerevisiae*, CAP interacts with multiple proteins and possesses multiple functional domains, suggesting a complex molecular function for this protein.

We described the binding of Cap1 with actin and its localization at the growing tip where the actin patch exists and at the septum where the actin ring is formed. This co-localization of Cap1 and actin supports the function of CAP as an actin sequestering protein, as previously observed in *S. cerevisiae* (Yu*, et al.*, 1999), as well as a protein that cooperates with cofilin to facilitate actin filament turnover (Moriyama & Yahara, 2002). Csh3 localized mainly in the cytoplasm in wild-type *S. pombe*, but it localized to the cell surface when *cap1* was deleted. This result suggests that the Csh3-Cap1 interaction is necessary for Csh3 localization in the cytoplasm.

We also observed that both Cap1 and Csh3 are able to form homodimers (or possibly multidimers). Dimerization of Cap1 occurs through both N.T. - N.T. and C.T. - C.T. interactions, while dimerization of Csh3 occurs only through N.T. - N.T. interactions (Fig. 8). The Csh3 protein has at least two domains that are involved in its interaction (i.e. one with Cap1 and one for dimerization). Cap1 has at least three domains that are involved in dimerization, and interaction with adenylyl cyclase, Csh3 and actin as summarized in Fig. 8.

In addition to the physical association between Cap1 and Csh3, we also observed a functional interaction between these proteins. Cell growth was retarded by over-expressing both *cap1* and *csh3*, but not *cap1* or *csh3* alone. The sensitivity of the *cap1* disruptant toward $CaCl_2$ was enhanced by concomitant disruption of *csh3*. Using a

construct expressing Csh3-GFP, we observed localization of Csh3 to the cytoplasm, but in the *cap1* deletion mutant, Csh3 was localized predominantly at the cell surface. Taken together, these observations indicate a functional interaction between Cap1 and Csh3, although an understanding of the physiological consequences of this interaction awaits further analysis. Recently, Csh3 was identified as the interacting protein of Eng2 which involves endocytosis (Encinar del Dedo, *et al.*, 2014). Csh3 co-localizes with Eng2 as cytoplasmic dots, although the structure of which is yet not clear. We did not observe the clear dots of Csh3 in wild type background, but intriguingly, we observed dots structure in *cap1* deletion background (Fig. 6). Some condition seems to affect the structure of Csh3 and lead to dots formation. As the involvement of CAP (SRV2) in *S. cerevisiae* has been shown (Wesp, *et al.*, 1997), our finding that Csh3 interacts with Cap1 and the finding of its interaction with Eng2 in *S. pombe* (Encinar del Dedo, *et al.*, 2014) may unveil a new regulatory network in endocytosis. Further analysis will be required in this respect.

$CaCl_2$ sensitivity of the *csh3*Δ *cap1*Δ strain is one of the interesting phenotypes we found in this study. A similar phenotype has been found in the *zds1* or *rad24* mutants in fission yeast (Yakura, *et al.*, 2006, Oowatari, *et al.*, 2009, Paul, *et al.*, 2009), and both of the genes are involved in sexual differentiation. The N-terminal domain of Cap1 is involved in sexual differentiation through the cAMP pathway (Kawamukai, *et al.*, 1991) and is also known to interact with Rad24 (Zhou, *et al.*, 2000). Together with the present study, showing its interaction with Csh3, these data suggest that the N-terminal domain of Cap1 is predominantly involved in the $CaCl_2$-related function, and suggests an interaction between Cap1 and a Ca^{2+} signaling pathway.

The actin-binding property of CAP is well-conserved among various eukaryotes, while its adenylyl cyclase-binding property is only found in two yeasts, and not in higher eukaryotes (Kawamukai unpublished data). The Csh3-binding property of CAP that we describe in this study is also not widely conserved, and we did not observe the binding of Csh3 with *S. cerevisia*e CAP. However, *S. cerevisiae* CAP does interact with the SH3 protein Abp1 (Lila & Drubin, 1997), and although this is not a Csh3 ortholog, this findings does suggest that the SH3-binding property of CAP may be conserved across multiple species. There are many SH3-containing proteins in *S. cerevisiae*, the most similar to Csh3 being Lsb1 (40% identity) and Lsb2 (36% identity), both of which also share the P/Q rich sequence that we found in Csh3. Lsb1 and Lsb2 are known to bind Las17/Bee1, a homolog of the Wiskott-Aldrich syndrome protein of mammals, and negatively regulate Las17 function (Madania, *et al.*, 1999, Spiess, *et al.*, 2013).

Orthologs of Csh3 have been found in other fungi such as *Candida, Pichia, Cryptococcus*, and *Aspergillus*, but no obvious orthologs have been found in plants and mammals. In *S. pombe*, the App1 protein (857 a.a.) shares greater similarity than Csh3 with the *S. cerevisiae* CAP-binding protein Abp1 (592 a.a.). Csh3 is a smaller protein than App1 and Abp1, and although App1 and Abp1 have the SH3 domain, these proteins do not have the P/Q rich sequence that was found in Csh3. Considering the differing activator proteins of adenylyl cyclase in both yeasts, namely Gα in fission yeast and RAS in budding yeast (Yamamoto*, et al.*, 2004, Ivey & Hoffman, 2005), it should not be surprising that there is some divergence in the CAP-associated proteins of these yeasts. While CAPs from higher eukaryotes also possess a presumable SH3 binding domain no binding protein with the SH3 domain has been identified so far, and no interaction between Csh3 and human Cap1 was observed in the present study. Further studies on the role of CAP in higher eukaryotes will be interesting, since we have observed both conserved and differentiated features in two highly divergent yeasts.

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Figure legend

Fig. 1. *S. pombe* Cap1 forms homodimers and interacts with actin. (A) *In vivo* dimerization of Cap1. Wild-type strain SP870 was co-transformed with a plasmid expressing GST or Cap1-GST under the *nmt1* promoter and the plasmid expressing GFP or Cap1-GFP under the *nmt1* promoter. Cells were grown in the absence of thiamine, and samples were prepared as in Materials and Methods. GST proteins were pulled down using glutathione-Sepharose beads, and GFP or Cap1-GFP in the precipitate was detected by immunoblotting with anti-GFP polyclonal antibody (right panel). An antibody against GST was used to detect GST fusion proteins (left panel). (B) *In vivo* binding of Cap1 to actin. Wild-type strain SP870 was co-transformed with a plasmid expressing GST or Cap1-GST under the *nmt1* promoter. Cells were grown in the absence of thiamine, and samples were prepared. GST proteins were precipitated using glutathione-Sepharose beads, and actin in the precipitate was detected by immunoblotting with anti-actin polyclonal antibody (right panel). An antibody against GST was used to detect GST fusion proteins (left panel). (C) Two-hybrid analysis of the interaction between Cap1 and actin. Yeast strain HF7c was co-transformed with a plasmid expressing actin fused to the GAL4 DNA-binding domain and a plasmid expressing the indicated Cap1 fragments fused to the GAL4 activator domain. Cells were spotted onto medium containing histidine (His+ plates), or onto medium lacking histidine (His- plates). If the GAL4 DNA-binding domain fusion protein and the GAL4 activator domain fusion protein interact with each other, the cells are able to grow on media lacking histidine.

Fig. 2. Two-hybrid analysis of the Cap1 domains involved in dimerization. (A) The N-terminal and the C-terminal regions of Cap1 interact with themselves. Yeast strain HF7c was co-transformed with a plasmid expressing full-length Cap1, or its N-terminal or C-terminal regions, fused to the GAL4 DNA-binding domain or the GAL4 activator domain. Vector and Sla1 (Tanabe*, et al.*, 2003, Tanabe*, et al.*, 2004) were used as negative controls. Cap1 segments included in the GAL4 activator domain are schematically shown on the right. Positive signals were detected as the histidine autotroph. (B) The dimerization domain in the N-terminal region of Cap1. Yeast strain HF7c was co-transformed with a plasmid expressing the full-length Cap1, or its N-terminal region, fused to the GAL4 DNA-binding domain, together with a plasmid

expressing various Cap1 fragments fused to the GAL4 activator domain and analyzed as in panel A. Cap1 segments included in the GAL4 activator domain are schematically shown on the right.

Fig. 3. Csh3 interacts with Cap1. *In vivo* binding of Csh3 to Cap1. A *cap1*Δ strain was co-transformed with a plasmid expressing GST or various Cap1-GST fusions under the *nmt1* promoter, together with a plasmid expressing GFP or Csh3-GFP under the *nmt1* promoter. Cells were grown in the absence of thiamine, and samples were prepared as described in Materials and Methods. GST fusion proteins were pulled down by glutathione-Sepharose beads, and GFP or Csh3-GFP in precipitates was probed by immunoblotting with anti-GFP polyclonal antibody. The arrowheads point to the GST-tagged Cap and its fragments.

Fig. 4. Csh3 can form homodimers. (A) Dimerization of Csh3 as detected by the two-hybrid test. Yeast strain HF7c was co-transformed with the plasmid expressing full-length Csh3, Csh3ΔN, or Csh3ΔSH3 fused to the GAL4 DNA-binding domain, together with a plasmid expressing full-length Csh3, Csh3ΔN, or Csh3ΔSH3 fused to the GAL4 activator domain. Cells were spotted onto medium containing histidine (His+ plates), or onto medium lacking histidine (His- plates). Vector and Sla1 were used as negative controls. Csh3 segments included in the GAL4 activator domain are schematically shown on the right. (B) *In vivo* dimerization of Csh3 detected by *in vivo* pull-down. Wild-type strain SP870 was co-transformed with a plasmid expressing GST or Csh3-GST under the *nmt1* promoter, together with a plasmid expressing GFP or Csh3-GFP under the *nmt1* promoter. Cells were grown in the absence of thiamine, and samples were analyzed by pull-down and Western blotting.

Fig. 5. Genetic and functional interaction between *csh3* and *cap1*. (A) Effect of over-expression of *csh3* and *cap1* on the growth of wild-type strains. Wild-type strain SP870 was co-transformed with a plasmid expressing Csh3 and a plasmid expressing Cap1. Empty vectors were used as controls. Cells were spotted onto PM minimal medium as serial dilutions (3 x 10^4 cells in the left row that are then diluted 5-fold in each subsequent spot on the right) and incubated at 25˚C for 6 days. (B) Effects of the deletion of Csh3 and Cap on cell growth. Various strains (wild-type, *csh3*Δ, *cap1*Δ and *csh3*Δ *cap1*Δ) were spotted onto YEA medium, PM minimal medium, or YEA medium

containing 0.1 M $CaCl_2$ as serial dilutions and incubated at 30˚C for 6 days.

Fig. 6. Intracellular localization of Csh3 and Cap1. Distributions of Csh3-GFP and Cap1-GFP fusion proteins were analyzed by direct fluorescence microscopy. The GFP fusion proteins were expressed under the *nmt1* promoters. Localization of Csh3-GFP and Cap1-GFP were observed in wild-type SP870 and *csh3*Δ and *cap1*Δ mutants. Localization of Cap1-GFP, Cap1ΔP1-GFP, Cap1ΔP2-GFP, and Cap1ΔP12-GFP were also observed in wild-type SP870 by fluorescence microscopy.

Fig. 7. Actin staining in *csh3*Δ, *cap1*Δ and *csh3*Δ *cap1*Δ strains. Cells were grown in YEA medium and stained with rhodamine-conjugated phalloidin. Cells were observed by fluorescence microscopy.

Fig. 8. Summary of domain functions in Cap1 and Csh3.
(A) The N-terminal region of Cap1 interacts with adenylyl cyclase, Csh3, and Cap1 itself, while the P2 region of Cap1 is important for localization of Cap1 in the growing end, and the C-terminal region of Cap1 interacts with actin and Cap1 itself. (B) The N-terminal region of Csh3 interacts with Csh3 and the SH3 region is important for Cap1 binding. HFD, helical folded domain; WH2, Wiskott-Aldrich-homology 2 domain; SH3, Src homology 3 domain; P/Q, proline/glutamine rich domain

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Materials and methods for supplementary Figures

Oligonucleotides used for sub-cloning are listed in Table S1. pBS-csh3-1 contains an *S. pombe* cDNA clone in pBluescript SK- (Stratagene). This was derived from the λZAPII clone and isolated from the *S. pombe* cDNA library (Kawamukai*, et al.*, 1991) using the *in vivo* excision procedure. The plasmid used for disruption of the *csh3* gene was constructed as follows. The DNA fragment containing the full-length *csh3* gene was amplified by PCR with pBS-csh3-1 as a template, using primers CSH3-2F and CSH3-2R. This PCR product was cloned in the *Not*I and *Xho*I sites of pBluescript SK-. The 1.8-kb *S. pombe ura4* cassette was then inserted into the *Hin*dIII site of this plasmid and the resulting construct was designated pBS-csh3::ura4.

The plasmid expressing Csh3ΔSH3, which lacks the central region (residues 126-213), including the SH3 domain of *csh3*, was constructed as follows. The fragment of *csh3* encoding the C-terminal region was amplified by PCR using primers CSH3-5F and T7, and pBS-csh3-3 as template, which contains *csh3* in the *Sma*I and *Bam*HI sites of pBluescript SK-. This PCR product was inserted into the *Hin*dIII - *Bam*HI digested fragment of pBS-csh3-3. The resulting construct was designated pBS-csh3ΔSH3 and was used as a template for PCR to amplify the fragment encoding Csh3ΔSH3.

The fragment of *cap1* encoding the full-length Cap1 protein was amplified by PCR using primers CAP2-hybF and CAP2-hybR. This PCR product was cloned in the *Sma*I and *Pst*I sites of pBluescript SK- to yield pBS-cap. pACL7 (Kawamukai*, et al.*, 1992) was used as a template for PCR to amplify the fragment encoding Cap1ΔP1, which lacked the central region (residues 297-367) including the proline-rich region P1.

The plasmid encoding Cap1ΔP2, which lacked the central region (residues 368-400), including the proline-rich region P2, was constructed as follows. The fragment of *cap1* encoding the C-terminal region was amplified by PCR using forward primer CAP-2F and reverse primer CAP2-hybR. This PCR product was inserted into the *Sna*BI - *Pst*I digested fragment of pBS-cap to yield pBS-capΔP2, which was used as a template for PCR to amplify the fragment encoding Cap1ΔP2. The plasmid expressing Cap1ΔP12, which lacks the central region (residues 297-400), including the proline-rich regions P1 and P2, was constructed as follows. The *cap1* fragment encoding the N-terminal region was amplified by PCR using primers CAP2-hybF and CAP-3R. This PCR product was inserted into the *Sma*I - *Sna*BI digested fragment of pBS-capΔP2 to yield pBS-capΔP12, which was used as a template for PCR to amplify the fragment encoding Cap1ΔP12.

The plasmids expressing various Csh3 proteins for the yeast two-hybrid method were

constructed as follows. Fragments of *csh3* encoding the full-length Csh3 protein or Csh3ΔSH3 were amplified by PCR using primers CSH3-1 and T7. The *csh3* fragment encoding Csh3ΔN, which lacked the N-terminal region (residues 1-120), was amplified by PCR using primers CSH3-3 and T7. Each PCR product was cloned into the *Sma*I and *Bam*HI sites of pBTM116, pGBT9, or pGAD424, and the resulting constructs were shown in Fig S1.

The plasmids expressing various Cap1 proteins for the yeast two-hybrid method were constructed as follows. Fragments of *cap1* encoding Cap1 (full-length), Cap1ΔP1, Cap1ΔP2, or Cap1ΔP12 were amplified by PCR using primers CAP2-hybF and CAP2-hybR. The fragment of *cap1* encoding $Cap1^{1-196}$ (residues 1-196) was amplified by PCR using primers CAP2-hybF and SpCAPNRev. The fragment of *cap1* encoding Cap1 N.T. (residues 1-296) was amplified by PCR using primers CAP2-hybF and CAP-3R. The fragment of *cap1* encoding Cap1 C.T. (residues 401-551) was amplified by PCR using primers CAP-2F and CAP2-hybR. Each PCR product was cloned in the *Sma*I and *Pst*I sites of pBTM116, pGBT9, or pGAD424, and the resulting constructs were shown in Fig.S1. pGAD424-cap1-69 was constructed by inserting the *Sma*I - *Sal*I digested fragment encoding $Cap1^{1-69}$ (residues 1-69) from pBS-cap into pGAD424. pGAD424-cap167-236 was constructed by inserting the *Eco*RI - *Dra*I digested fragment encoding $Cap1^{167-236}$ (residues 167-236) from pBS-cap into the *Eco*RI and *Sma*I sites of pGAD424. pGAD424-cap167-296 was constructed by inserting the *Eco*RI - *Pst*I digested fragment encoding $Cap1^{167-296}$ (residues 167-296) from pBTM116-cap N.T. into pGAD424.

The plasmid expressing GFP in *S. pombe* was constructed as follows. The fragment of the GFP (S65A) gene was amplified by PCR using primers GFP-F and GFP-R. This PCR product was cloned in the *Not*I and *Sal*I sites of pSLF172 and pSLF172L (Forsburg & Sherman, 1997), and the resulting constructs were designated pSLF172U-GFP and pSLF172L-GFP (Ozoe*, et al.*, 2002), respectively. pSLF172L and pDS472L were derived from pSLF172 and pDS472a (Forsburg & Sherman, 1997) by converting the *ura4* marker to the *LEU2* marker, respectively.

The plasmids that express GFP- or GST-fused protein were constructed as follows. Various fragments of *csh3* encoding Csh3 (full-length) or Csh3ΔSH3 were amplified by PCR using forward primer CSH3-2F and reverse primer CSH3-4R. Various fragments of *cap1* encoding Cap1 (full-length), Cap1ΔP1, Cap1ΔP2, and Cap1ΔP12 were amplified by PCR using primers CAP-1F and CAP-1R. The fragment of *cap1* encoding $Cap1^{238-551}$ (residues 238-551) was amplified by PCR using primers CAP-5F and

CAP-1R. The fragment of *cap1* encoding Cap1 C.T. (residues 401-551) was amplified by PCR using primers CAP-6F and CAP-1R. Each PCR product was cloned in the *Not*I site of pSLF172U-GFP, pSLF172L-GFP, pDS472a, or pDS472L. The resulting constructs expressing GFP- or GST-fused proteins were shown in Fig. S1.
pDS473a-GSTCAPNT expressing Cap1$^{1-236}$-GST (residues 1-236) was previously described (Zhou*, et al.*, 2000). pARTN1-csh3 that over-expresses *csh3* under the control of the *adh1* promoter was constructed by inserting the *Not* I digested fragment encoding full-length Csh3 protein from pBS-csh3-1 into pARTN1 that was derived from pART1 (Kawamukai*, et al.*, 1992) by insertion of a *Not*I linker in the *Sma*I site. pACU1 that over-expresses *cap1* under the control of the *adh1* promoter was constructed by inserting the *Not*I digested DNA fragment encoding Cap1 from pCAP7-3 (Kawamukai*, et al.*, 1992) into pAUN1, which was derived from pARTN1 by converting the *LEU2* marker to the *ura4* marker.

Fig. S1. Restriction map of the *csh3* locus. (A) Restriction sites on the insert of pBS-csh3-1. The arrow indicates the coding region and direction of *csh3*. The point of the *ura4* insertion in the fragment that was used to construct the *csh3* disruption is shown below. (B) Restriction maps of the plasmids used in this study. Open boxes and shaded boxes indicate coding sequences of *csh3* and *cap1*, respectively. The solid box indicates the *ura4* gene and hatched boxes indicate the promoters in each plasmid. Stippled boxes indicate LexA, GFP, or GST. The flat lines indicate non-coding sequences of the *csh3* or *cap1* locus. The faint wavy lines indicate vector sequences.

Fig. S2. The predicted protein sequence encoded by *csh3*. (A) Comparison of the amino acid sequences of Csh3 and *S. cerevisiae* Lsb1 and Lsb2. Identical amino acids are highlighted. Amino acid residues are numbered on the right. The spaces were manually introduced to maximize the alignment. (B) Schematic structures of Csh3 and its *S. cerevisiae* homologues. Stippled boxes and Hatched boxes indicate SH3 domains and P/Q domains, respectively. The degree of identity between the C-terminal regions of the homologues and Csh3 is indicated.

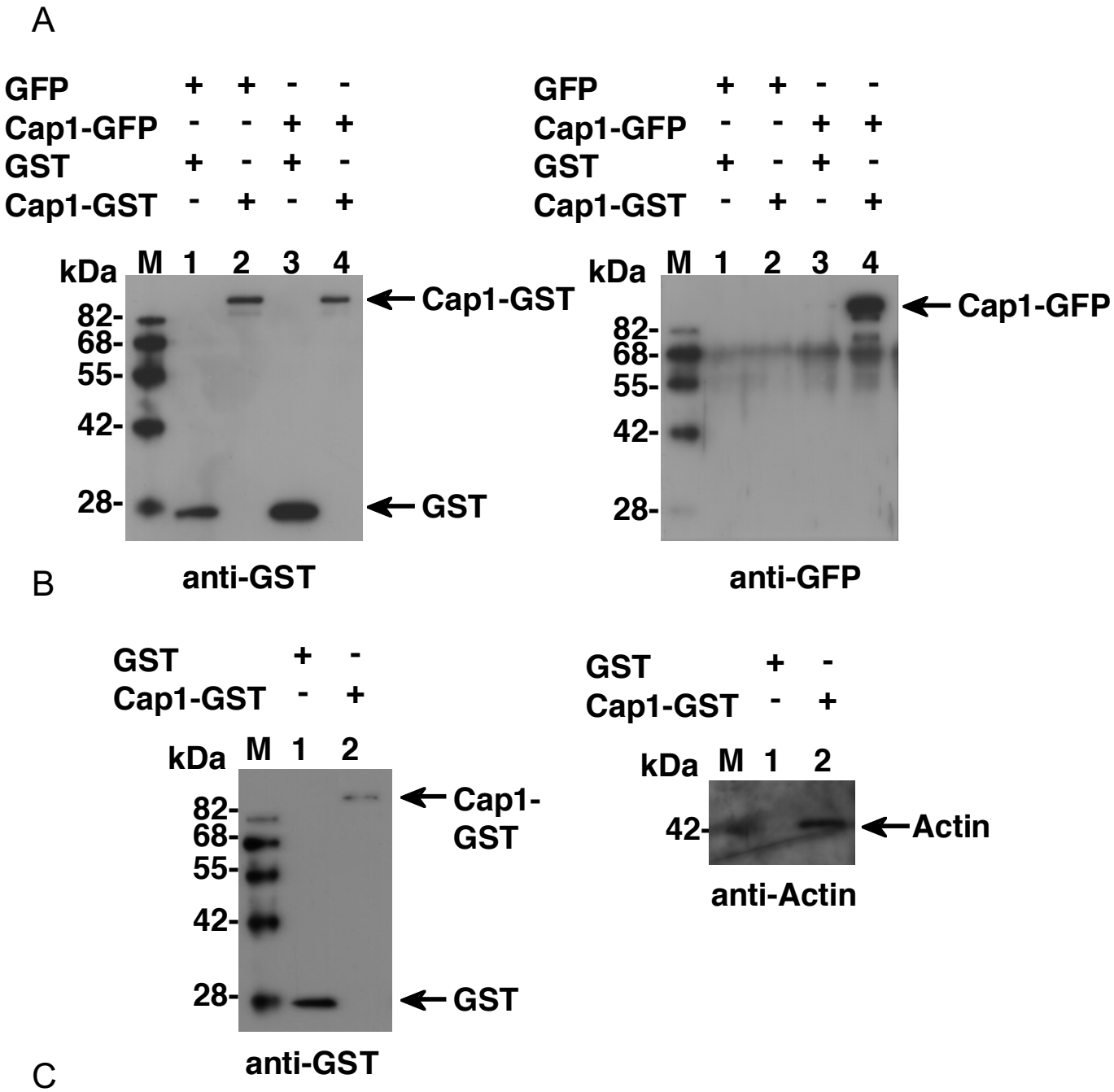

A
GFP + + - -
Cap1-GFP - - + +
GST + - + -
Cap1-GST - + - +
kDa M 1 2 3 4
82-
68-
55-
42-
28-
Cap1-GST
GST
anti-GST
GFP + + - -
Cap1-GFP - - + +
GST + - + -
Cap1-GST - + - +
kDa M 1 2 3 4
82-
68-
55-
42-
28-
Cap1-GFP
anti-GFP
B
GST + -
Cap1-GST - +
kDa M 1 2
82-
68-
55-
42-
28-
Cap1-GST
GST
anti-GST
GST + -
Cap1-GST - +
kDa M 1 2
42-
Actin
anti-Actin
C
1 2 3 4 5 6 7 8 9 10
His+
1 2 3 4 5 6 7 8 9 10
His-
1. vector
2. Sla1
3. Cap1 1-69
4. Cap1 167-236
5. Cap1 167-296
6. Cap1 167-368
7. Cap1 167-412
8. Cap1 401-531
9. Cap1
10. vector

Fig. 1

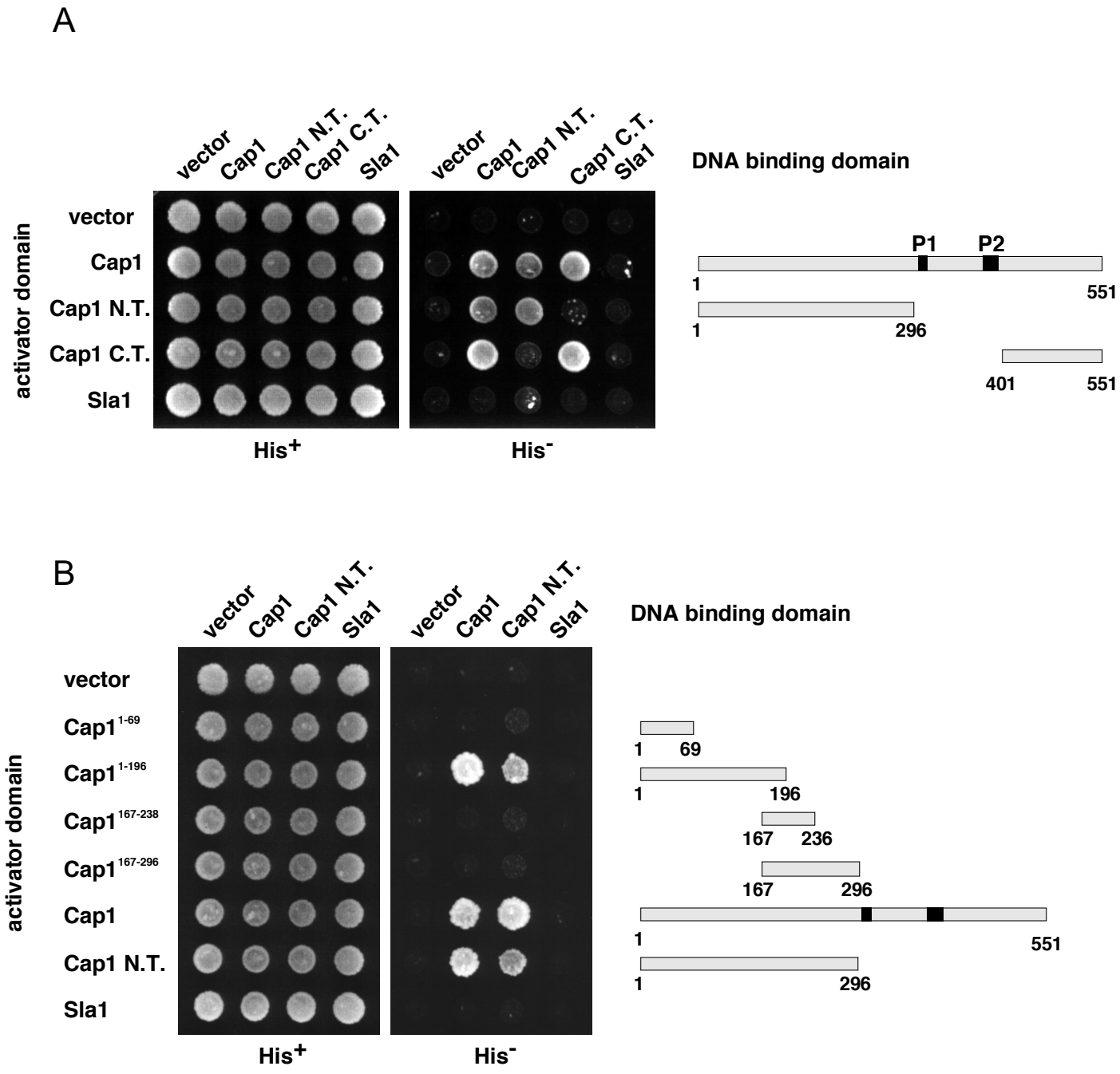

A
vector
Cap1
Cap1 N.T.
Cap1 C.T.
Sla1
activator domain
His+
His-
DNA binding domain
P1
P2
1
551
296
401
B
Cap1 1-69
Cap1 1-196
Cap1 167-238
Cap1 167-296
69
196
236

Fig. 2

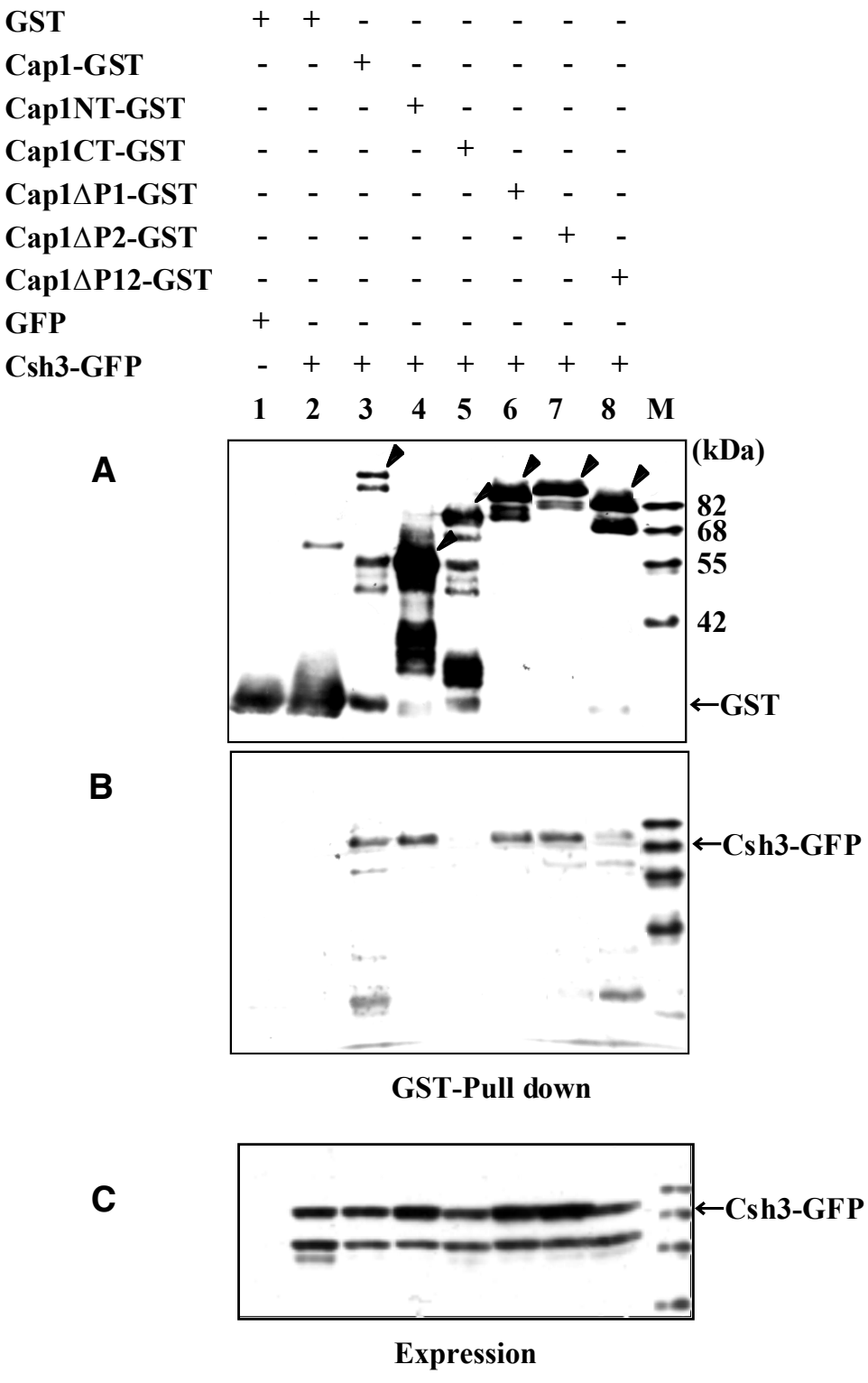

GST + + - - - - - -
Cap1-GST - - + - - - - -
Cap1NT-GST - - - + - - - -
Cap1CT-GST - - - - + - - -
Cap1ΔP1-GST - - - - - + - -
Cap1ΔP2-GST - - - - - - + -
Cap1ΔP12-GST - - - - - - - +
GFP + - - - - - - -
Csh3-GFP - + + + + + + +
1 2 3 4 5 6 7 8 M
A
(kDa)
82
68
55
42
←GST
B
←Csh3-GFP
GST-Pull down
C
←Csh3-GFP
Expression

Fig. 3

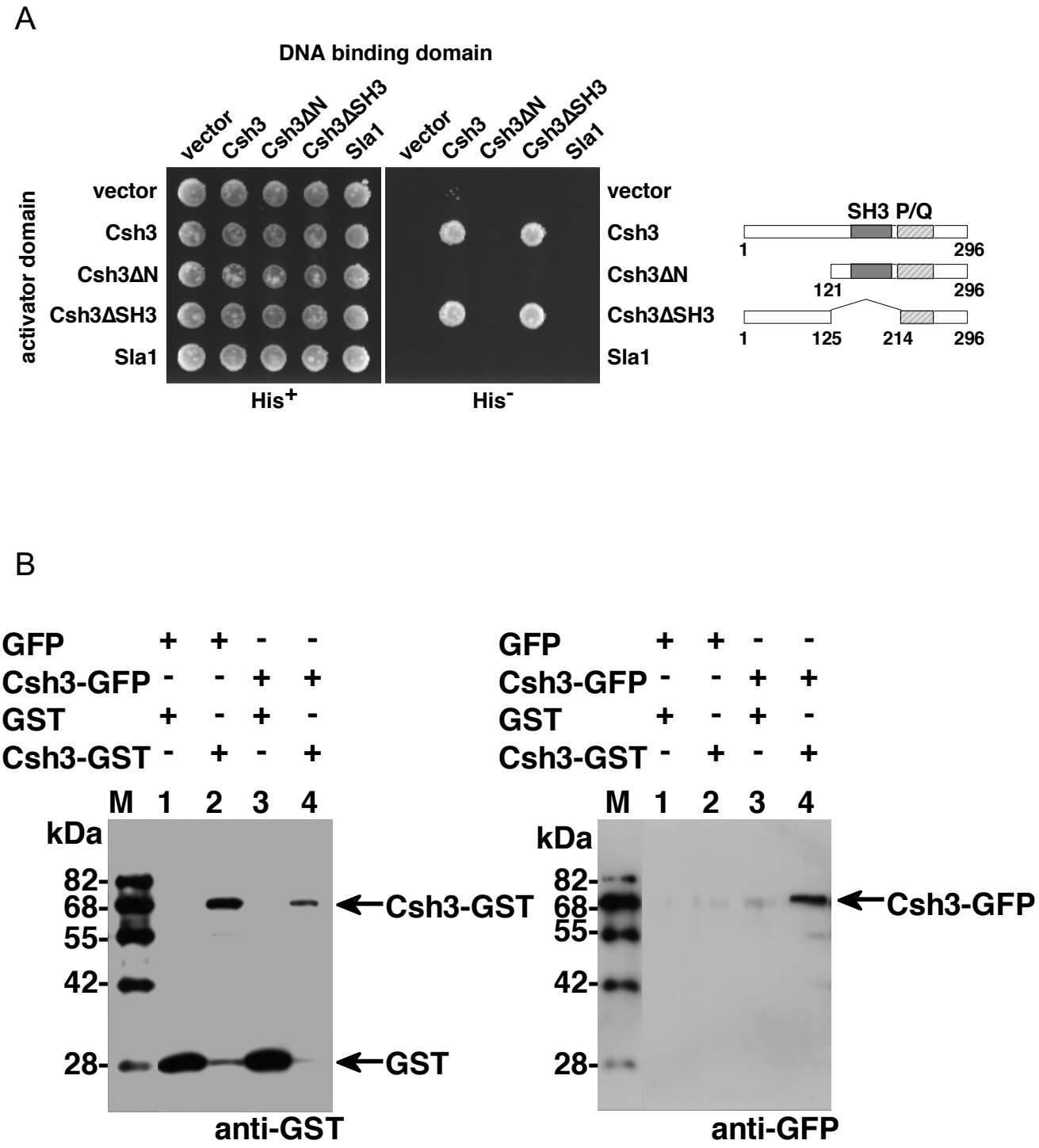
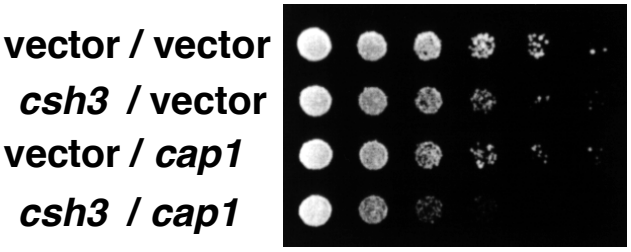

A
DNA binding domain
vector
Csh3
Csh3ΔN
Csh3ΔSH3
Sla1
activator domain
His+
His-
SH3 P/Q
1
296
121
125
214
B
GFP
Csh3-GFP
GST
Csh3-GST
M
kDa
82-
68-
55-
42-
28-
Csh3-GST
GST
anti-GST
Csh3-GFP
anti-GFP

Fig. 4

A


vector / vector
csh3 / vector
vector / cap1
csh3 / cap1

B

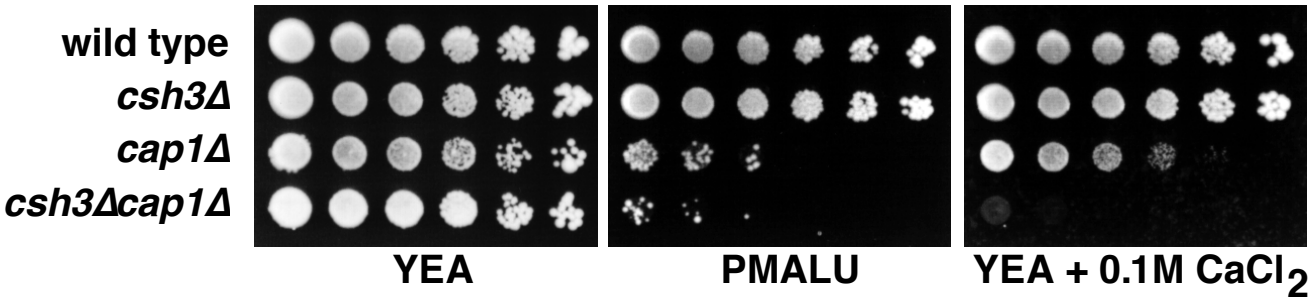
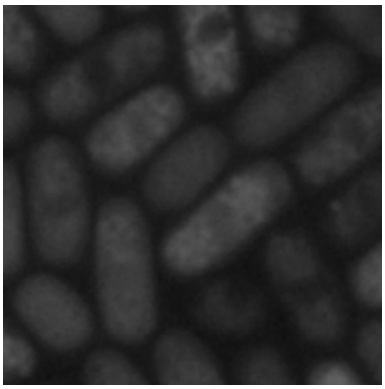
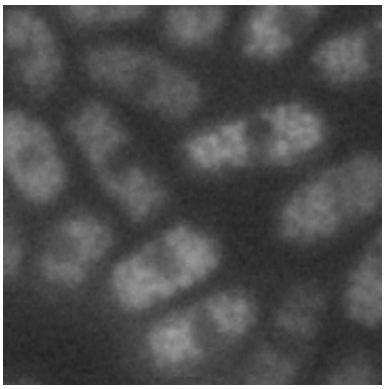

wild type
csh3Δ
cap1Δ
csh3Δcap1Δ
YEA
PMALU
YEA + 0.1M CaCl2

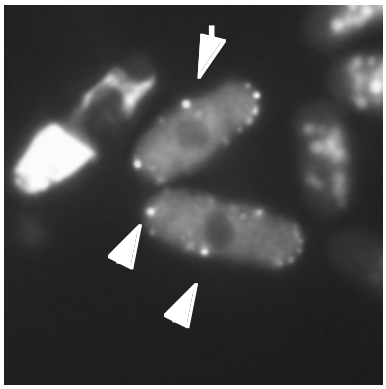
Fig. 5



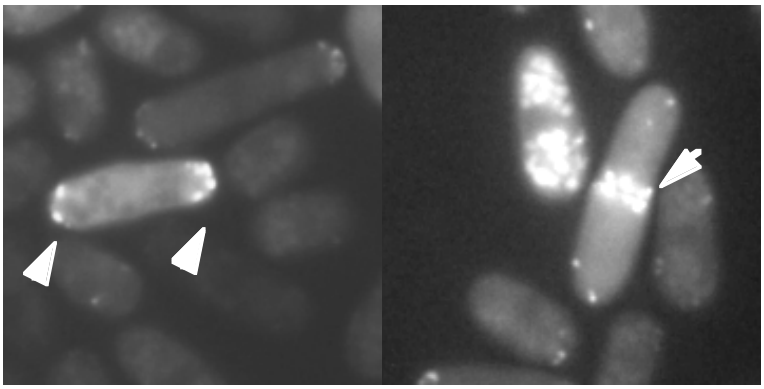
GFP/SP870



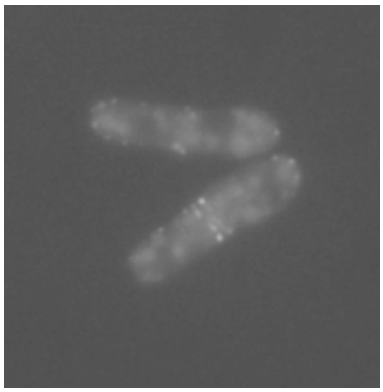
Csh3-GFP/SP870



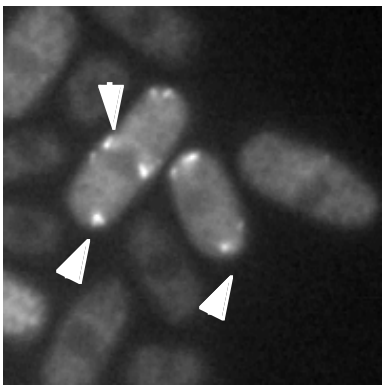
Csh3-GFP/*cap1Δ*



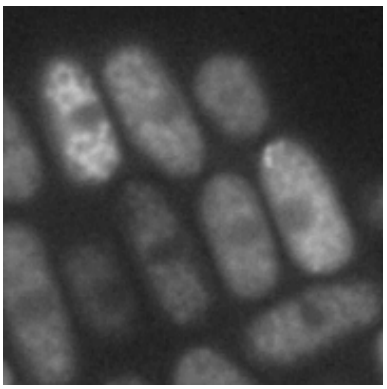
Cap1-GFP/SP870



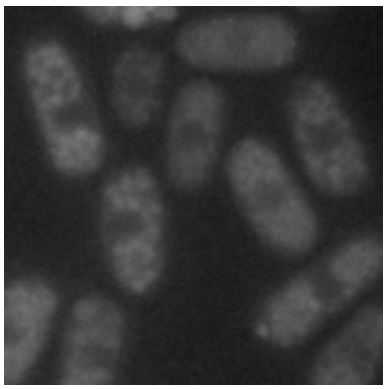
Cap1-GFP/*csh3Δ*



Cap1ΔP1-GFP/SP870

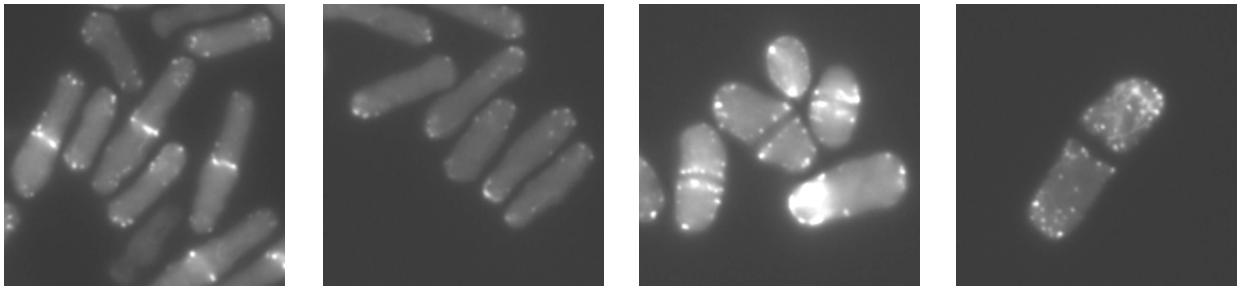


Cap1ΔP2-GFP/SP870



Cap1ΔP12-GFP/SP870

Fig. 6



SP870 ***csh3Δ*** ***cap1Δ*** ***csh3Δ cap1Δ***

Fig. 7

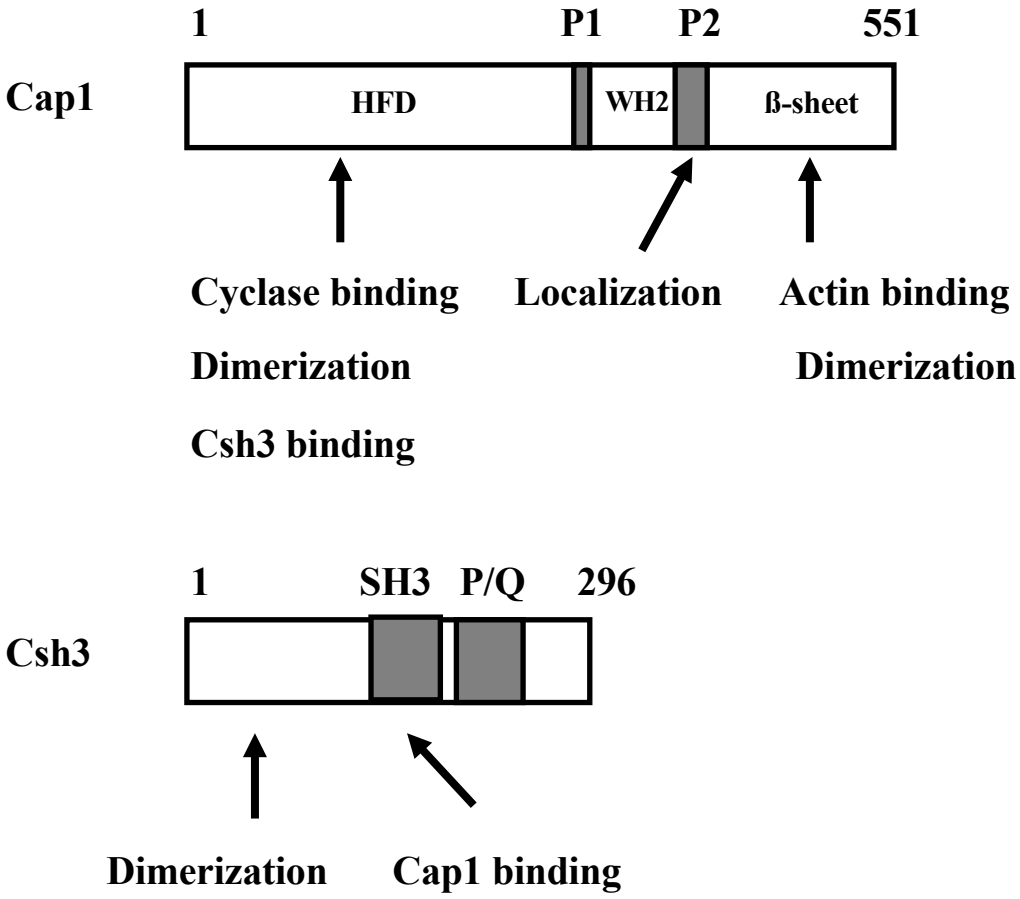

1
P1
P2
551
Cap1
HFD
WH2
ß-sheet
Cyclase binding
Dimerization
Csh3 binding
Localization
Actin binding
Dimerization
1
SH3
P/Q
296
Csh3
Dimerization
Cap1 binding

Fig. 8

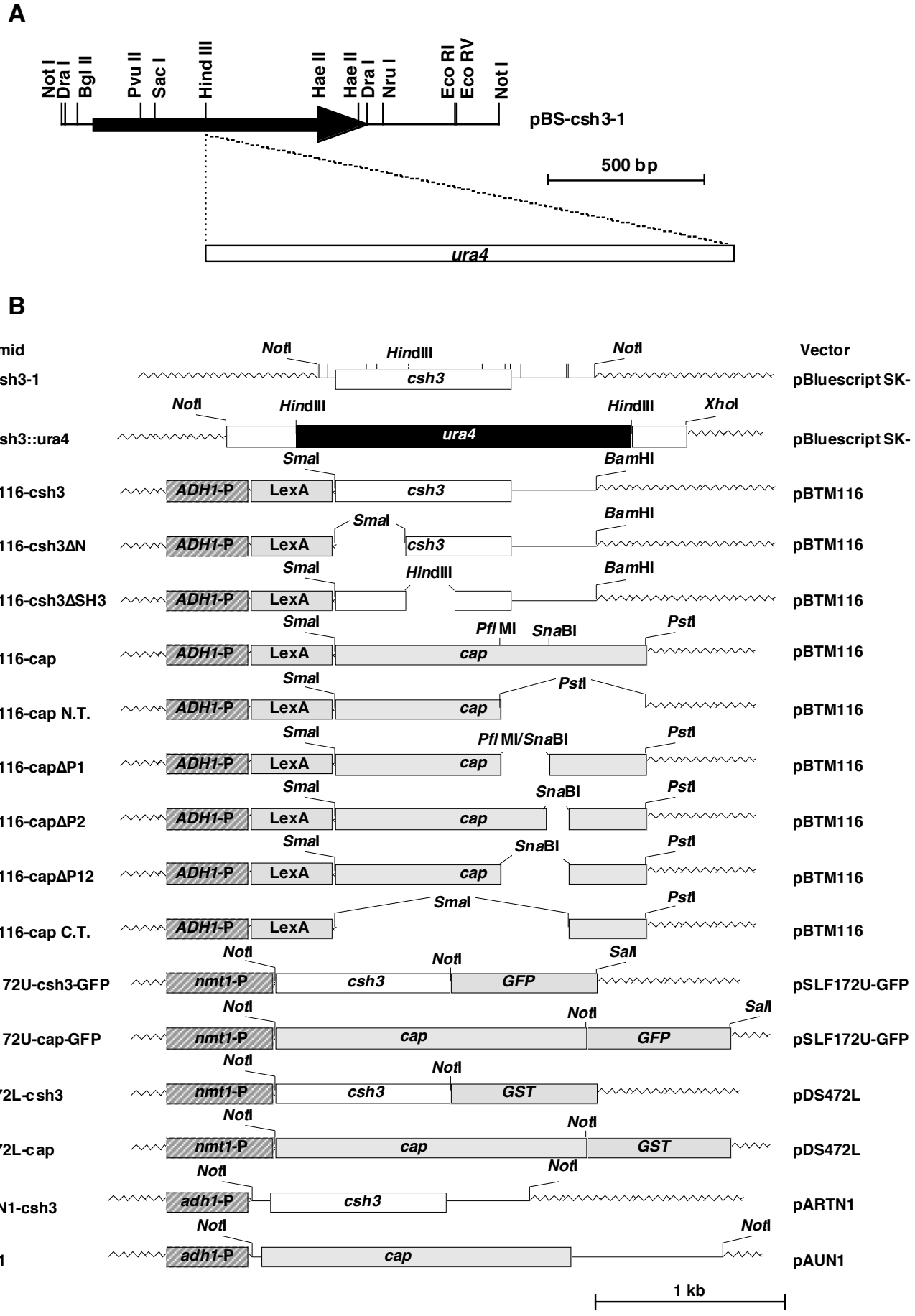

A
Not I
Dra I
Bgl II
Pvu II
Sac I
Hind III
Hae II
Hae II
Dra I
Nru I
Eco RI
Eco RV
Not I
pBS-csh3-1
500 bp
ura4
B
Plasmid
Vector
pBS-csh3-1
NotI
HindIII
NotI
csh3
pBluescript SK-
pBS-csh3::ura4
NotI
HindIII
HindIII
XhoI
ura4
pBluescript SK-
pBTM116-csh3
SmaI
BamHI
ADH1-P
LexA
csh3
pBTM116
pBTM116-csh3ΔN
SmaI
BamHI
csh3
pBTM116
pBTM116-csh3ΔSH3
SmaI
HindIII
BamHI
pBTM116
pBTM116-cap
SmaI
PflMI
SnaBI
PstI
cap
pBTM116
pBTM116-cap N.T.
SmaI
PstI
cap
pBTM116
pBTM116-capΔP1
SmaI
PflMI/SnaBI
PstI
cap
pBTM116
pBTM116-capΔP2
SmaI
SnaBI
PstI
cap
pBTM116
pBTM116-capΔP12
SmaI
SnaBI
PstI
cap
pBTM116
pBTM116-cap C.T.
SmaI
PstI
pBTM116
pSLF172U-csh3-GFP
NotI
NotI
SalI
nmt1-P
csh3
GFP
pSLF172U-GFP
pSLF172U-cap-GFP
NotI
NotI
SalI
nmt1-P
cap
GFP
pSLF172U-GFP
pDS472L-csh3
NotI
NotI
nmt1-P
csh3
GST
pDS472L
pDS472L-cap
NotI
NotI
nmt1-P
cap
GST
pDS472L
pARTN1-csh3
NotI
NotI
adh1-P
csh3
pARTN1
pACU1
NotI
NotI
adh1-P
cap
pAUN1
1 kb

Fig. S1

A

```
Csh3    MDHKNYLNHV IRGIYNDFQF LVDEGVVERS ALDWVHANIH LQDGPASPVT APAAQPVESS VPLPLPKRKS  70

Csh3    SVEKRAGSVA SAVAAMSLSQ NSGEKRTPEE PRKLPGVPAP QKQSEASSVN SSTEKLPPPP SYPGPNTAHK 140
Lsb1                        MSASLVNRSL KNIRNELEFL KESNVISGDI FELINSKLPE KWDGNQRSPQ  50
Lsb2                        MSASLINRSL TNIRTELDFL KGSNVISNDV YDQINKSLPA KWDPANAPRN  50

Csh3    NV----ERVL AMYDFPGPDA GDLGFHAGEV IIVLEHVNND WWRGELNGKE GIFPSNYVR- ---------- 195
Lsb1    NADTE-EYVE ALYDFEAQQD GDLSLKTGDK IQVLEKISPD WYRGKSNNKI GIFPANYVKP AFTRSASPKS 119
Lsb2    ASPASLEYVE ALYQFDPQQD GDLGLKPGDK VQLLEKLSPE WYKGSCNGRT GIFPANYVKP AFSGSNGPSN 120

Csh3    -LLEDSAVKA QPPPPPPQQN YPP------- --AASSS--- ------APPM QYQQTAYPPQ QA----PYPP 242
Lsb1    AEAASSSTVS RPSVPPP--S YEP------- --AASQYPSQ QVSAPYAPPA GYMQ-APPPQ QQQAPLPYPP 177
Lsb2    L--------- ---PPPPQYK AQELQQIPTQ NSAASS---- ---------- -YQQQ----- ------PFPP 152

Csh3    -VQAYPQAPQ QPI------- -VVAQPTEHK H-SSTFKKIG SGLGSAFVFG AGATAGADLV NSIF.      296
Lsb1    PFTNYYQQPQ QQYAPPSQQA PVEAQPQQSS GASSAFKSFG SKLGNAAIFG AGSAIGSDIV NSIF.      241
Lsb2    PSTNYYQQPQ QQ-PQQAPPP QQQQQQQQHQ SSHSHLKSFG SKLGNAAIFG AGASIGSDIV NNIF.      215
```

B

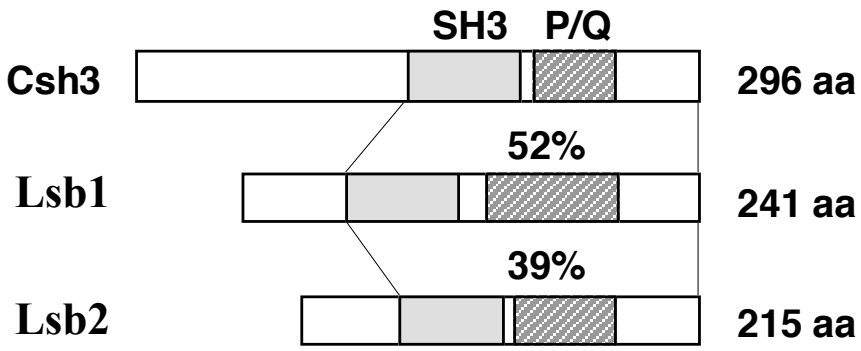


Fig. S2