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COP9 signalosome components play a role in the mating pheromone response of *S. cerevisiae*

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A family of genetically and structurally homologous complexes, the proteasome lid, Cop9 signalosome (CSN) and eukaryotic translation initiation factor 3, mediate different regulatory pathways. The CSN functions in numerous eukaryotes as a regulator of development and signaling, yet until now no evidence for a complex has been found in *Saccharomyces cerevisiae*. We identified a group of proteins, including a homolog of Csn5/Jab1 and four uncharacterized PCI components, that interact in a manner suggesting they form a complex analogous to the CSN in *S. cerevisiae*. These newly identified subunits play a role in adaptation to pheromone signaling. Deletants for individual subunits enhance pheromone response and increase mating efficiency. Overexpression of individual subunits or a human homolog mitigates *sst2*-induced pheromone sensitivity. Csi1, a novel CSN interactor, exhibits opposite phenotypes. Deletants also accumulate Cdc53/cullin in a Rub1-modified form; however, this role of the CSN appears to be distinct from that in the mating pathway.

INTRODUCTION

The Cop9 signalosome (CSN) is an eight-component complex, which is apparently found in all multicellular eukaryotes as a regulator of signaling and developmental processes (Bech-Otschir *et al.*, 2002; Chamovitz and Glickman, 2002). Mutations in CSN subunits cause plant seedlings grown under darkness to mimic light development growth, while *Drosophila* mutants do not develop beyond larval stage (Chamovitz *et al.*, 1996; Oron *et al.*, 2002). The CSN has been implicated both in the phosphorylation of numerous regulatory proteins, such as Jun and p53 (Seeger *et al.*, 1998; Naumann *et al.*, 1999; Bech-Otschir *et al.*, 2001), and in the removal of Nedd8/Rub1 ubiquitin-like modification from the cullin subunit of E3 ubiquitin ligases (Lyapina *et al.*, 2001; Zhou *et al.*, 2001). Significant structural and genetic similarities are shared between the CSN, lid and eukaryotic translation initiation factor 3 (eIF3) (Glickman *et al.*, 1998; Kapelari *et al.*, 2000; Fu *et al.*, 2001; Glickman and Ciechanover, 2002). All CSN and lid subunits contain one of two signature motifs: the PCI (proteasome, CSN, eIF3) or MPN (Mpr1, Pad1 N-terminal) domains (Aravind and Ponting, 1998; Hofmann and Bucher, 1998; Maytal-Kivity *et al.*, 2002). So far, no evidence for a complex in *Saccharomyces cerevisiae* has been produced, and straightforward identification of the PCI-motif subunits has been unsuccessful, leading to the assumption that CSN orthologs are not present in this organism (Aravind *et al.*, 2000).

The paradigm of cell-to-cell communication is the mating response in yeast. Haploid cells respond to pheromone by arresting in G_1, forming polarized extensions (shmoos), and eventually fusing with their partner to yield a diploid zygote (Elion, 2000; Dohlman, 2002). Pheromone excreted by one mating type cell binds to a G protein-coupled cell-surface receptor of an opposite mating type. The resulting conformational change causes $G\alpha$ to bind GTP and release $G\beta\gamma$, which in turn activates the mitogen-activated protein kinase (MAPK) signaling pathway and the synthesis of proteins necessary for the mating process. Desensitization is a vital property of any signaling pathway. Cells that respond to pheromone too easily will arrest prematurely and will be out-competed by cells that do go on dividing as usual. Sst2 is a potent regulator of G-protein signaling (RGS) that desensitizes the mating signal by activating the GTPase activity of free $G\alpha$, allowing it to rebind $G\beta\gamma$ (Elion, 2000; Dohlman, 2002). We describe proteins resembling CSN subunits in *S. cerevisiae* that also participate in the mating pathway by desensitizing the signal and augmenting adaptation to pheromone exposure.

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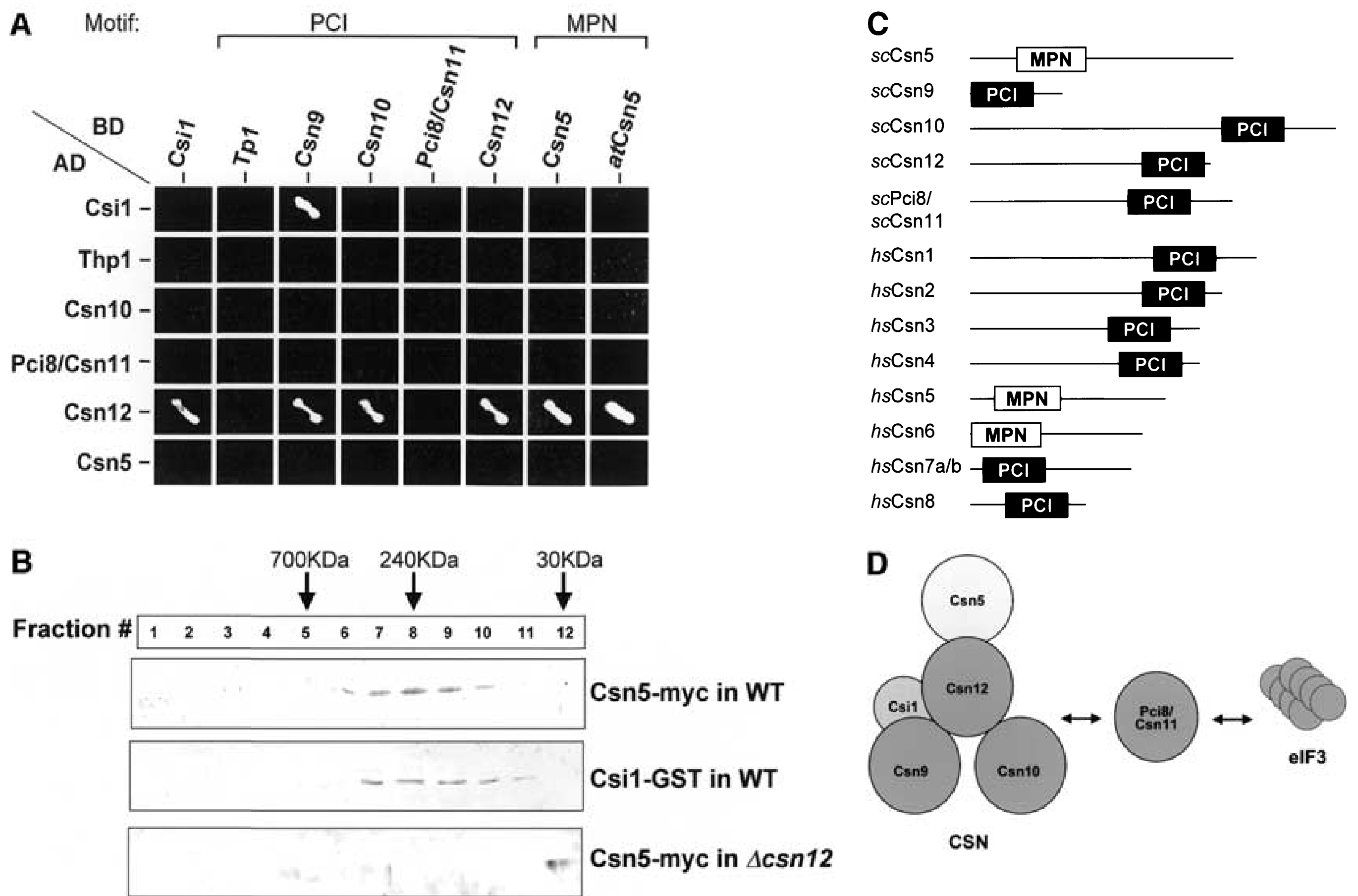

Fig. 1. Identification of CSN-like components in *S. cerevisiae*. (**A**) Y2H. Pairwise interactions of budding yeast PCI proteins (Csn9, Csn10, Csn12, Pci8/Csn11, Thp1), an MPN protein (Csn5/Rri1) and Csi1. Growth on -ade-his selective media indicates positive interactions. Positive interactions are depicted in (D). Csn5 from plant, *at*Csn5, is included for comparison. (**B**) Comigration. Glycerol gradient fractionation of yeast cell extract containing naturally abundant tagged Csn5 or Csi1 in wild-type (WT) or Δ*csn12* backgrounds. Csn5 and Csi1 comigrate in a complexed form with an apparent molecular weight of 240 kDa. In Δ*csn12*, Csn5-myc is detected only in the lowest molecular weight fraction. (**C**) Structural comparison of identified CSN subunits from budding yeast and human. Cartoons are drawn to scale and depict the signature PCI and MPN domains. See also Supplementary data. (**D**) Possible model for a CSN-like complex in budding yeast. Csn5, Csn9, Csn10 and Csn12 co-interact with Csn12 playing a pivotal role in the structural organization of this complex (PCI, dark gray; MPN, light gray). Only interactions identified in (A) and (B) are specified. An additional PCI protein, Pci8/Csn11, interacts both with the CSN and eIF3 and may be a shared subunit. A non-PCI/non-MPN protein, Csi1, interacts with Csn9 and Csn12.

RESULTS AND DISCUSSION

Identification of a CSN in budding yeast

In an attempt to determine whether functional homologs of CSN subunits exist in *S. cerevisiae*, we used the 'generalized profile method' (Hofmann, 2000) to scan the genome for orphaned PCI proteins (see Supplementary data available at *EMBO reports* Online). When tested for pairwise interactions, three previously uncategorized PCI proteins (Csn9, Csn10 and Csn12) interact with each other and with a protein homologous to Csn5 (Figure 1A). Importantly, plant and yeast Csn5 maintain identical interaction patterns with other yeast CSN candidates. Furthermore, Csn5, Csn9, Csn10 and Pci8/Csn11 coprecipitate together, indicating that they are complexed (Gavin *et al.*, 2002). For comparison, an additional PCI protein, Thp1, is involved in transcription elongation but has not been assigned to any known PCI complex (Gallardo and Aguilera, 2001) and does not interact with the newly identified CSN subunits (Figure 1A). Other PCI-containing proteins such as lid components Rpn3, Rpn5, Rpn6, Rpn7 and Rpn12 or the eIF3 subunits Rpg1/eIF3a and Nip1/eIF3c are essential and classified as components of known PCI complexes. We included in our screen a protein termed Csi1 (CSN interactor 1) that contains neither a PCI nor an MPN domain but can coprecipitate with members of this complex (Uetz *et al.*, 2000; Gavin *et al.*, 2002). Csi1 is found to interact with Csn9 and Csn12 (Figure 1A).

Endogenously expressed Csn5-6myc migrates in a complexed form with an apparent molecular weight of ~240 kDa (Figure 1B). Upon deletion of *csn12*, Csn5 migrates consistent with its monomeric molecular weight, indicating that incorporation of Csn5 into the complex is dependent on Csn12. Endogenous Csn12 fractionates at ~240 kDa as well (data not shown). This role for Csn12 in maintaining the integrity of the CSN complex is supported by its y2h interactions (Figure 1A). Similarly in plants, *at*Csn5 is also incorporated into the CSN only in the

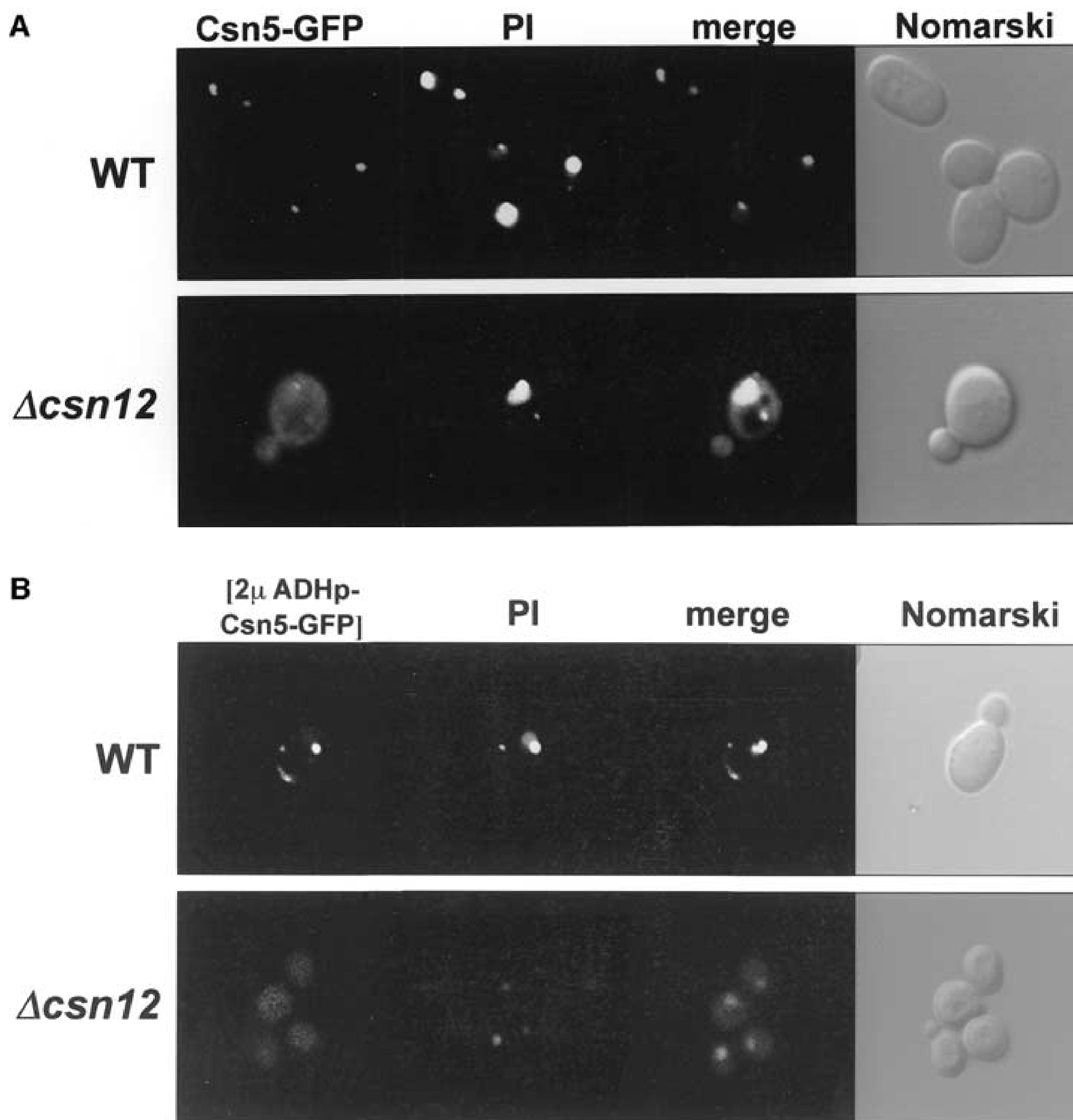

Fig. 2. Cellular localization of Csn5 in *S. cerevisiae*. (**A**) Localization of natural abundance Csn5. Logarithmically growing cells expressing naturally abundant GFP-tagged Csn5 were monitored by confocal microscopy. Images show Csn5–GFP fluorescence, propidium iodide-stained nuclei (PI), overlay of the two channels, and a Nomarski optical image. Csn5 is concentrated in the nucleus in wild-type (WT) but is delocalized throughout the cell in absence of Csn12. (**B**) Localization of overexpressed Csn5. Images are as in (A), but strains were transformed with multicopy plasmids expressing GFP-tagged Csn5 under the ADH promotor. Overexpressed Csn5 is similarly localized to the nucleus with slight cellular localization; in Δ*csn12*, Csn5 is delocalized throughout the cell.

presence of *at*Csn1 (Kwok *et al*., 1998). As Csi1 does not contain the signature PCI or MPN domains, we tested whether it too fractionates with the CSN. Csi1–GST comigrates with Csn5 in similar molecular weight fractions (Figure 1B).

Four PCI proteins (Csn9, Csn10, Csn11 and Csn12), one MPN protein (Csn5) and one interactor (Csi1) co-interact in *S. cerevisiae*, whereas the CSN in other eukaryotes forms a 6+2 complex (Figure 1C and D). Despite low sequence homology, dendrogram analysis points to a common evolutionary ancestor of all CSN subunits from yeast and other species (see Supplementary data). Pci8/Csn11 has also been identified as an interactor of the eIF3 complex and a possible homolog of Int6/eIF3e (Shalev *et al*., 2001), suggesting that it may be a shared subunit. Association of eIF3e with the CSN also occurs in plants and mammals (Yahalom *et al*., 2001; Hoareau *et al*., 2002), though the mechanistic meaning of this dual interaction is still unclear. The combined molecular weight of these 'core' subunits is calculated to be ~250 kDa, in agreement with their migration pattern, suggesting that they form a complex. We cannot preclude possible dimerization of this putative complex, especially as Csn12 interacts with itself, or association of yet additional subunits or interactors (CSIs).

Properties of the CSN

Cellular localization of endogenously expressed GFP-tagged Csn5 is predominantly nuclear (Figure 2A). Nuclear localization is largely maintained upon overexpression (Figure 2B). Deletion of *csn12* causes dramatic delocalization of Csn5 throughout the cytoplasm in either case (Figure 2A and B). Nuclear localization has been documented for Csn5 in other species, while compromising complex integrity promotes delocalization of Csn5 in plants (Kwok *et al.*, 1998; Seeger *et al.*, 1998; Mundt *et al.*, 2002). Together, we advocate a nuclear complex with possible cytoplasmic localization for certain monomers or subcomplexes.

Deletions of *csn5*, *csn9*, *csn10*, *pci8/csn11* or *csi1* bring about accumulation of Rub1-modified Cdc53/cullin, confirming that the CSN partakes in cullin modification in budding yeast (Figure 3A). While this paper was under review, these results were independently confirmed (Wee *et al.*, 2002), and evidence that Csn5/Rri1 is the catalytic subunit was demonstrated (Cope *et al.*, 2002). Interestingly, Cdc53 modification is insignificantly altered in Δ*csn12*, which promotes complex disassociation, indicating that integrity of the entire complex is not essential for this hydrolase activity (Figure 3A). Upon overexpression of any individual subunit, however, the extent of Rub1 conjugation is indistinguishable from wild-type (WT; Figure 3B), suggesting that no subunit is independently responsible for Rub1-hydrolase activity. That Csn2 is also essential for cullin deNeddylation in mammalian cells supports a requirement for multiple CSN subunits in deNeddylation (Yang *et al.*, 2002). Quite possibly, removal of Rub1 from Cdc53 may be carried out by (Csn5-containing) subcomplexes or a tightly associated enzyme.

The CSN is involved in regulating pheromone response

Befitting a developmental role, deletions of *csn5*, *csn9* or *csn12* show increased mating efficiency by up to 100% compared with WT (Figure 4A). Not all subunits behave similarly; deletions of *csn10* or *pci8* show little or no effect on mating, and Δ*csi1* is appreciably poorer at mating than WT (Figure 4A). This effect on mating is linked to greater pheromone sensitivity; most CSN deletants form shmoos quicker and to a larger extent than WT (Figure 4B). Again, Δ*csi1* displays an opposite phenotype, distinguishing it from the MPN or PCI members of this complex. Unique phenotypes for individual CSN subunits are documented in other organisms as well (Mundt *et al.*, 2002; Oron *et al.*, 2002).

Unable to recover from pheromone-induced cell cycle arrest, Δ*sst2* cells form a halo of no-growth around the source of pheromone (Figure 4C). Overexpression of Csn9 or Csn10 in Δ*sst2* allow for numerous cells to adapt and resume growth within this halo (Figure 4C). A strong effect is also observed upon expression of *hs*Csn1, underscoring a conserved role for PCI members of the CSN. Conversely, deletion of *csi1* facilitates adaptation of Δ*sst2* cells, whereas deletions of CSN subunits (especially Csn5 or Csn9) enhance sensitivity (Figure 4D). It appears that CSN components promote adaptation to pheromone by mitigating G protein-coupled signaling. Interestingly, Csi1 appears to reverse the effect of the CSN on the mating pathway. If so, Csi1 is the first recognized regulator of the CSN in any organism. A link between the CSN and G protein signaling is supported by interaction of Csn9 with Gpa1 (Uetz *et al.*, 2000) and suppression of *gpa1* mutations by *hs*Csn1 (Spain *et al.*, 1996).

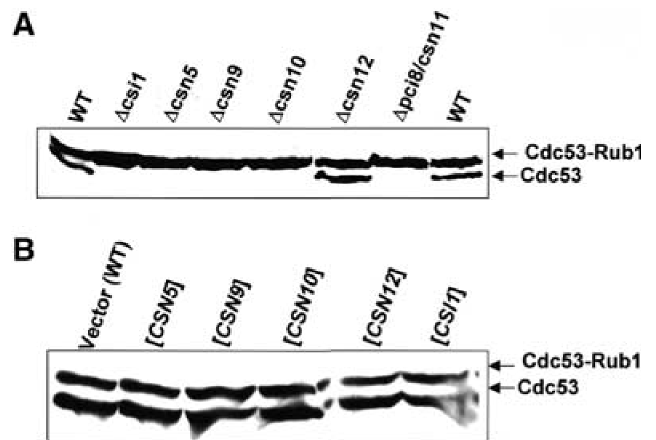

Fig. 3. The role of CSN in Cdc53 modification. (**A**) CSN deletants. Total protein extracts of wild-type (WT) or *csn* deletants were probed with anti-Cdc53 to visualize the extent of Cdc53 modification by Rub1. Cdc53 accumulates almost exclusively in a Rub1-modified form upon deletion of newly identified CSN-like subunits, with the exception of Δ*csn12*. (**B**) Overexpression. Modification of Cdc53 by Rub1 was probed in extracts from cells overexpressing each of the CSN subunits. Cdc53–Rub1 conjugation levels upon overexpression of any CSN subunit are indistinguishable from WT.

It is unclear whether the role of the CSN in the mating response stems from its documented properties. Deletions of most CSN subunits enhance pheromone response and increase Rub1–Cdc53 conjugation levels. However, *csn12* null also enhances mating efficiency yet shows no effect on Cdc53 modification, whereas Δ*csi1* exhibits an opposite effect on the mating pathway yet accumulates Rub1-modified Cdc53. Notably, overexpressions of individual PCI subunits do not appear to significantly influence levels of Cdc53 modification, yet they decrease pheromone sensitivity, providing evidence for a role of individual subunits in regulating mating but not in hydrolytic removal of Rub1. Together, these results suggest that the role of the CSN in the pheromone response of *S. cerevisiae* is uncoupled from its role in Cdc53 modification.

G protein-coupled cell surface receptors mediate response to light, small chemicals and hormones. Sst2^{RGS} is considered to be the predominant factor for desensitizing the Gpa1$^{G\alpha}$ signal in yeast, yet we show that individual CSN subunits can override *sst2* deletion. Thus, the CSN negatively regulates this pathway either by aiding in Gα recycling or by repressing downstream components of the MAPK cascade. The conserved function of *hs*Csn1 in the pheromone response of yeast (Spain *et al.*, 1996), together with the role of CSN in AP-1 signaling (Naumann *et al.*, 1999; Tsuge *et al.*, 2001), suggests that some of the developmental roles of the CSN may also be executed via repression of MAPK pathways.

METHODS

Bioinformatics. Iterative database searches were performed with a non-redundant data set using profiles calculated from previously published alignments (Hofmann and Bucher, 1998) for

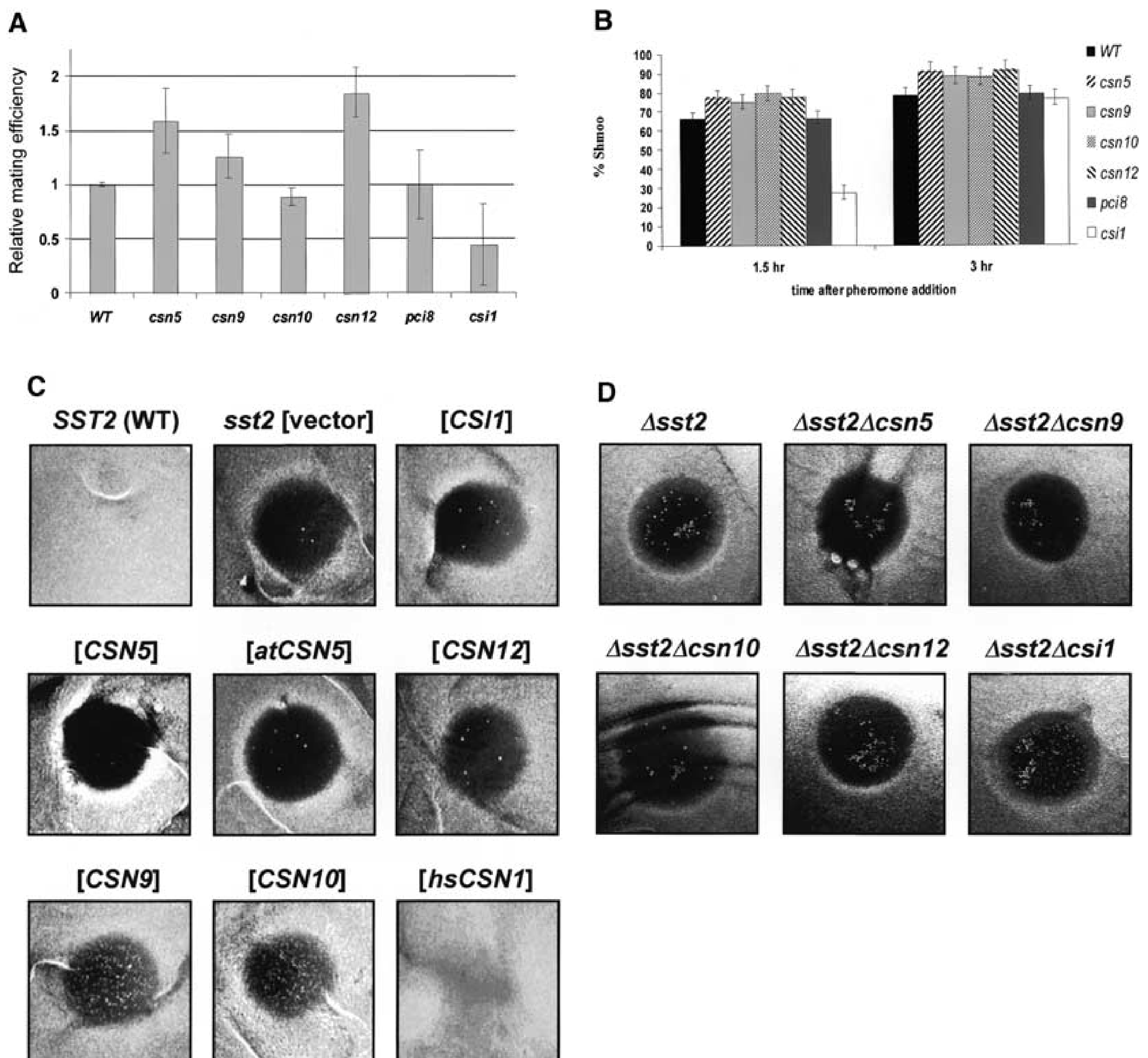

Fig. 4. Role of CSN subunits in the mating pheromone pathway. (**A**) Mating efficiency of CSN disruptions. Deletants in *csn5* or *csn12* show increased mating efficiency by up to 100% when mated with wild-type (WT) cells. Δ*csn10* or Δ*pci8* show little or no effect on mating, while *csi1* null is appreciably poorer at mating compared with WT. Error bars are calculated from an average of 5–7 independent mating experiments for each strain. (**B**) Shmoo formation. Exponentially growing *MATa* cultures were treated with 1 mM α-factor pheromone. After 1.5 and 3 h, aliquots were fixed and the percentage of cells with shmoo-like projections were counted. Error bars represent the average of triplicate 200-cell samples. Cells deleted for *csn5*, *csn9*, *csn10* or *csn12* respond to pheromone quicker and to a greater extent than WT cells. Deletion of *csi1* delays the response to pheromone exposure. (**C**) Adaptation to pheromone response (halo assay). Cultures of Δ*sst2* cells containing a vector plasmid (control) or overexpressing individual CSN subunits were plated on selective media and treated with α-factor to the center of the plate. A halo of no-growth encircles the pheromone due to the inability of *sst2* cells to exit G_1 arrest. Formation of spontaneous colonies within this halo in Δ*sst2* culture is at single digits (vector). Overexpression of Csn9, Csn10 or *hs*Csn1 enhances adaptation, allowing for numerous cells to resume growth within the halo and form colonies. (**D**) Adaptation to pheromone CSN deletants. As in (C) except that Δ*sst2* and double deletant strains were plated on complete media. Deletion of *csi1* facilitates adaptation to pheromone exposure. Thick growth of Δ*sst2*Δ*csi1* occurs at the periphery of the halo, where local concentration of pheromone is lower, and numerous colonies are noticeable throughout the halo. Deletions of other CSN subunits have a mild negative effect on adaptation, decreasing the number of viable colonies compared with the Δ*sst2* single deletion.

detection of additional MPN and PCI domains in the *S. cerevisiae* genome. See Supplementary data for details and results.

CSN subunits. Rri1 is encoded by YDL216c and is referred to as Csn5 according to the accepted nomenclature (Deng *et al.*, 2000). Due to the lack of a discernible ortholog relationship with characterized CSN subunits 1–8 from other species (see Supplementary data), the suffixes 9, 10, 11 and 12 were used for the putative CSN-like subunits encoded by YDR179c, YOL117w,

PCI8/YIL071c and YJR084w, respectively. Csi1 is encoded by YMR025w.

Strains and plasmids. WT and deletant strains were purchased from EUROSCARF. Double mutants were made by mating haploids, sporulation and tetrad dissection. Relevant genes were cloned by PCR and inserted into CEN plasmids (YCplac111). For overexpression under the ADH2 promotor, open reading frames were cloned into YIplac128. GFP-, GST- or Myc-tagged versions of CSN subunits were constructed in the same plasmids. Based on fluorescence intensity of GFP-tagged proteins, overexpression is ~10-fold. Eliezer Lifshits and John Colicelli generously provided plasmids with plant *CSN5* and *hsCSN1*, respectively

Yeast 2-hybrid. Coding regions were inserted into the Matchmaker III yeast 2-hybrid plasmids pGADT7 or pGBKT7 (Clontech). Positive interactions were tested in strain AH109 (Clontech) by Ade and His autotrophy. None of the constructs shown in Figure 1A permitted growth on -ade-his media when transformed alone.

Glycerol gradients. Total yeast cell extract (0.5 ml) expressing tagged CSN subunits was separated on a 15–40% glycerol gradient in 100 mM NaCl, 50 mM Tris–HCl pH 7.4 and protease inhibitor cocktail (Roche) for 20 h at 30 000 r.p.m. with an SW41 rotor. Gradient fractions (1 ml) were resolved by SDS–PAGE and immunoblotted. For anti-Cdc53 immunoblots (yc-17; Santa Cruz), extract was prepared by mechanical lysis in 50 mM trichloroacetic acid after rinsing with NaF, 10 mM Tris buffer pH 7.4, 0.02% Na-azide and protease inhibitors.

Fluorescence microscopy. Cells were washed with 70% v/v ethanol and incubated with 0.4 mg/ml RNase A in 50 mM sodium citrate pH 7 for 2 h and transferred to 50 mM sodium citrate pH 7, 10 mM NaCl, 0.1% NP-40 and 5 μg/ml propidium iodide (PI). Reconstructions of GFP and PI channels were made using an MRC-1024 laser confocal scanning microscope (Bio-Rad) with the objective Nikon Plan Apo 603/1.40.

Shmoo formation. Exponentially growing cells (OD_{600} = 0.5) were treated with 1 μM final concentration of α-factor pheromone (Sigma). Aliquots were fixed in 7.4% formaldehyde and 0.15 M NaCl. Triplicate batches of 200 cells each were counted by optical microscope for percentage of shmoos.

Mating assay. Logarithmic cultures of mutant and WT of opposite mating type were incubated at a ratio of 1:10 for 3 h at 30°C and plated on selective media to determine the number of diploid cells. Assays were carried out such that at least 200 diploid cells would be counted in each experiment. The number of haploid cells in each test strain was determined by growth on selective media for the limiting strain.

Halo assays. Three hundred microliters of exponentially growing culture (OD_{600} = 0.5) of Δ*sst2 MAT**a*** strains were plated on selective media (for CSN overexpressions) or complete minimal media (for *csn* deletions) supplemented with 0.1 M citrate, pH 4.5, and 5 μl of 50 μM α-factor was added to the center of the plate. Growth was photographed after 3 days at 30°C.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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