Interaction of the yeast γ -tubulin complex-binding protein Spc72p with Kar1p is essential for microtubule function during karyogamy

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The spindle pole body component Kar1p has a function in nuclear fusion during conjugation, a process known as karyogamy. The molecular role of Kar1p during this process is poorly understood. Here we show that the yeast γ -tubulin complex-binding protein Spc72p interacts directly with the N-terminal domain of Kar1p, thereby targeting the γ -tubulin complex to the half bridge, a substructure of the spindle pole body, where it organizes microtubules. This binding of Spc72p to Kar1p has only a minor role during vegetative growth, whereas it becomes essential for karyogamy in mating cells, explaining the important role of Kar1p in this process. We also show that the localization of Spc72p within the spindle pole body changes throughout the cell cycle and even more strongly in response to mating pheromone. Taken together, these observations suggest that the relocation of Spc72p within the spindle pole body is the 'landmark' event in the pheromone-induced reorganization of the cytoplasmic microtubules.

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Introduction

In budding yeast, microtubule organizing functions are provided by the spindle pole body (SPB), a multi-layered structure that is embedded in the nuclear envelope throughout the cell cycle (Byers and Goetsch, 1975). The SPB organizes two main sets of microtubules. The cytoplasmic outer plaque and the nuclear inner plaque are SPB substructures that are associated with the cytoplasmic and nuclear microtubules, respectively. An additional SPB substructure that functions in cytoplasmic microtubule (CM) organization is the half bridge, a one-sided extension of the central plaque layered on top of the nuclear envelope (Byers and Goetsch, 1975). Nuclear and cytoplasmic microtubules are attached to the inner and outer plaque of the SPB throughout the cell cycle (Byers and Goetsch, 1975; illustrated in Figure 8A). In contrast, CMs originate only in G₁ of the cell cycle from the SPB half bridge (Byers and Goetsch, 1975). During vegetative growth the

nuclear microtubules are involved in SPB separation, spindle formation and chromosome segregation (Jacobs *et al.*, 1988), whereas the CMs function in SPB orientation, spindle positioning and in migration of the nucleus into the bud (Palmer *et al.*, 1992; Carminati and Stearns, 1997; Shaw *et al.*, 1997).

Upon the exposure of yeast cells to mating pheromone the CMs reorganize such that they are now directed from the half bridge of the SPB towards the mating projection (Byers and Goetsch, 1975; Brachet *et al.*, 1998). Following this microtubule reorganization, a and α cells fuse, the two nuclei are moved together along the interdigitating microtubules and finally fuse, a step known as karyogamy (reviewed by Rose, 1996). Genes involved in karyogamy have been identified by genetic screens (Conde and Fink, 1976; Kurihara *et al.*, 1996). Kar1p is particularly interesting since it is found at the half bridge of the SPB (Spang *et al.*, 1995) and functions in both karyogamy (Conde and Fink, 1976; Vallen *et al.*, 1992) and SPB duplication (Rose and Fink, 1987). However, little is known about the molecular role of Kar1p in karyogamy and its possible involvement in the pheromone-induced rearrangement of the CMs at the SPB.

How microtubules form *in vivo* became clearer with the discovery of γ -tubulin as a universal component of centrosomes and SPBs that is involved in microtubule formation (Oakley and Oakley, 1989; Oakley *et al.*, 1990; Stearns *et al.*, 1991; Stearns and Kirschner, 1994). γ -tubulin is found as part of a large complex (Zheng *et al.*, 1995) containing γ -tubulin, homologues of yeast Spc98p and Spc97p (Rout and Kilmartin, 1990; Geissler *et al.*, 1996; Knop *et al.*, 1997; Martin *et al.*, 1998; Murphy *et al.*, 1998; Tassin *et al.*, 1998) and additional proteins, depending on the organism (Zheng *et al.*, 1995; Murphy *et al.*, 1998). In budding yeast the γ -tubulin or Tub4p complex consists of the γ -tubulin, named Tub4p, and the SPB components Spc98p and Spc97p (Rout and Kilmartin, 1990; Geissler *et al.*, 1996; Knop *et al.*, 1997; Knop and Schiebel, 1997, 1998). Like other γ -tubulin complexes (Stearns and Kirschner, 1994; Moudjou *et al.*, 1996), the Tub4p complex assembles in the cytoplasm (Pereira *et al.*, 1998) and is then targeted to the sites of microtubule organization at the SPB via the binding of Spc97p and Spc98p to the N-terminal domain of Spc72p (Knop and Schiebel, 1998) and of Spc110p (Knop and Schiebel, 1997; Nguyen *et al.*, 1998). Spc72p is associated with the cytoplasmic outer plaque, whereas Spc110p is located with the nuclear inner plaque, explaining how the Tub4p complex is bound to the cytoplasmic and nuclear sides of the SPB (Rout and Kilmartin, 1990; Spang *et al.*, 1996; Knop *et al.*, 1997; Wigge *et al.*, 1998). The term γ -tubulin complex-binding protein (GTBP) has been coined for proteins such as Spc72p and Spc110p that target γ -tubulin complexes to distinct cellular localizations (Knop and Schiebel, 1998).

How Spc110p is embedded within the SPB is relatively well understood. Spc110p has been purified in a complex containing yeast calmodulin (Cmd1p), and the SPB components Spc29p and Spc42p (Knop *et al.*, 1997). Spc42p forms a layer within the centre of the SPB (Bullitt *et al.*, 1997) to which the C-terminus of Spc110p is anchored via Spc29p (Elliott *et al.*, 1999). Cmd1p binds to a site in the C-terminus of Spc110p (Geiser *et al.*, 1993; Stirling *et al.*, 1994) which is adjacent to the Spc29p-binding domain and evidence has been obtained that Cmd1p binding to Spc110p may regulate the interaction of Spc110p with Spc29p (Elliott *et al.*, 1999). In contrast, the binding proteins of Spc72p at the SPB have not been identified. However, it is known that the C-terminus of Spc72p is sufficient to target this protein to the SPB (Knop and Schiebel, 1998).

How the CMs of the half bridge are organized in G₁ of the cell cycle and in mating cells is largely unknown. Here we provide the first evidence that the Tub4p complex-binding protein Spc72p interacts directly with the N-terminal domain of the half bridge component Kar1p, explaining the function of Kar1p in conjugation. By means of this interaction the Tub4p complex is targeted to the half bridge where it organizes CMs. We also provide evidence that the interaction of Kar1p with Spc72p has only a minor function during vegetative growth, but becomes essential for CM organization during conjugation.

Results

The C-terminus of Spc72p interacts with the N-terminal domain of Kar1p

KAR1 was identified in a genetic screen for mutants with a defect in karyogamy (Conde and Fink, 1976) and although Kar1p has been localized to the SPB half bridge (Spang *et al.*, 1995), its function during karyogamy has remained elusive. Using the yeast two-hybrid system, we found that amino acids 116–236 of Kar1p interact with the C-terminus of the Tub4p-complex-binding protein Spc72p (Spc72p^{231–622}; Figure 1A). This interaction was found to be specific, because it was drastically affected by the *kar1-1* and *kar1-Δ15* mutations (Figure 1B, note the strongly reduced β-galactosidase activity of *kar1-1*+*C-SPC72* and *kar1-Δ15*+*C-SPC72* compared with *KAR1*+*C-SPC72*) which strongly reduce karyogamy efficiency (Conde and Fink, 1976; Vallen *et al.*, 1992). Controls established that the *Gal4-HA-KAR1* gene fusions were expressed similarly (Figure 1C).

To verify the interaction between Kar1p and Spc72p, we expressed *KAR1*, *kar1-1*, *kar1-Δ15* and *SPC72*^{231–622} as glutathione *S*-transferase (GST) or histidine-tagged gene fusions in *Escherichia coli* and tested whether Spc72p^{231–622} can interact directly with Kar1p. Binding of Spc72p^{231–622} to GST–Kar1p was observed (Figure 1D, lane 2, asterisk), and this interaction was reduced at least 10-fold by the P150S missense mutation in Kar1-1p (lane 4). Little binding of Spc72p^{231–622} to Kar1-Δ15p (lane 3), and no interaction with GST (lane 1) was detectable. In addition, Kar1p bound directly to Spc72p^{231–622} *in vitro* when both purified proteins were incubated (data not shown).

Genetic proof for a functional relationship between *SPC72* and *KAR1* during conjugation came from the

partial suppression of the karyogamy defect of *kar1-1* cells through high gene dosage of *SPC72* (Figure 1E). The ratio of zygotes to diploids was low when wild-type cells were crossed with each other (bar 2) and this value was not affected by *SPC72* on plasmid pRS426 (bar 1). This reflects the fact that during mating of wild-type cells the two cell bodies and the nuclei fuse to give a zygote which then multiplies during the course of the experiment and thereby gives rise to diploid cells which again divide. In contrast, *kar1-1* cells are karyogamy deficient when crossed to wild-type and the progenies of such a zygote are haploid containing one of the two parental nuclei (reviewed by Rose, 1996). Therefore, the ratio of zygotes to diploids is high (bar 4, *kar1-1* cells with the control plasmid pRS426). This deficiency of *kar1-1* cells was in part suppressed by *SPC72* on the multi-copy plasmid pRS426 (bar 3). Taken together, these data show that Kar1p interacts directly with Spc72p.

***In vivo* binding of Spc72p to the SPB half bridge is dependent on the N-terminal domain of Kar1p**

Spc72p was shown to be a component of the outer plaque (Knop and Schiebel, 1998; Wigge *et al.*, 1998) and evidence has been obtained that it is also associated with the half bridge (Souès and Adams, 1998; Adams and Kilmartin, 1999). To test whether the localization of Spc72p with the half bridge depends on Kar1p *in vivo*, we made use of the *kar1-Δ15* mutation (Vallen *et al.*, 1992), which lacks the Spc72p-binding domain of Kar1p (illustrated in Figure 3A). Cells of *kar1-Δ15* do not show an obvious phenotype during vegetative growth, however, they are deficient in karyogamy during conjugation, caused by a CM organization defect (Vallen *et al.*, 1992). Spc72p was found to be associated with the SPB in wild-type cells (Figure 2B, 1 and 2) and in cells of *kar1-Δ15* (Figure 2B, 5–7, red and white arrows) during vegetative growth throughout the cell cycle. However, upon the addition of α-factor (Figure 2B, 5–7, *kar1-Δ15*, black arrow in 5; note that α-factor arrested and vegetatively growing cells were mixed to allow a direct comparison of the signals) Spc72p was no longer detectable at the SPB of *kar1-Δ15* cells, whereas it was still associated with the SPB of pheromone-treated wild-type cells (Figure 2B, 3 and 4, white arrows in 4). We established by immunoblotting with anti-N-Spc72p antibodies that the cellular levels of Spc72p were similar in *KAR1* and *kar1-Δ15* cells with or without α-factor (data not shown).

The Kar1p-dependent association of Spc72p with the SPB in mating cells was confirmed in zygotes. Two *SPC72-GFP* cells of opposite mating type were incubated and the Spc72p-GFP signals were followed over time. The cell bodies fused and before karyogamy two Spc72p-GFP signals were observed, each associated with the SPB of the two nuclei (Figure 2A, upper). Upon longer incubation the two nuclei and the SPB fused (data not shown). In contrast, when a Concanavalin A Texas-Red-labelled *SPC72-GFP* wild-type cell was mated with an unlabelled *SPC72-GFP kar1-Δ15* cell (Figure 2A, lower right, black arrow) the two nuclei did not fuse, even after a prolonged incubation, due to the karyogamy defect caused by the *kar1-Δ15* mutation (Vallen *et al.*, 1992). Importantly, only the SPB of the wild-type cell carried a

Fig. 1. The C-terminal domain of Spc72p interacts with the N-terminus of Kar1p. (A) Kar1p and Spc72p interact in the yeast two-hybrid system. Plasmid pACT2 (no insert) or pACT2 with the indicated *KAR1* fragments (Gal4-fusions) were co-transformed with pEG202 (no insert) or pEG202 containing *SPC72* (LexA-fusions) into strain SGY37. The activity was determined by a plate assay. Blue colony colour indicates interaction. (B) Kar1-1p and Kar1-Δ15p are defective in their interaction with Spc72p. *KAR1* (codons 1–408) in pACT2 and *SPC72* derivatives in pEG202 were transformed into cells of SGY37. ‘Control’ indicates that the plasmid pEG202 was co-transformed with pACT2–*KAR1*. *C-SPC72* contains codons 231–622 of *SPC72*. β-galactosidase activity of lysed cells was measured as described in Materials and methods. Note, it is unclear why the pACT2–*C-SPC72* construct gave a stronger signal than pACT2–*SPC72*. (C) It was established by immunoblotting with anti-HA antibodies that the *Gal4-HA-KAR1* (lane 1), *Gal4-HA-kar1-Δ15* (lane 2) and *Gal4-HA-kar1-1* (lane 3) gene fusions were expressed to similar levels. (D) Binding of recombinant histidine-tagged C-Spc72p to GST–Kar1p. GST (lane 1), GST–Kar1p (lane 2), GST–Kar1-Δ15p (lane 3) and GST–Kar1-1p (lane 4) proteins bound to glutathione–Sepharose were incubated with an *E.coli* extract containing 6His–C-Spc72p^{231–622} (lane 5). Bound proteins were analysed by SDS–PAGE and Coomassie Blue staining, and immunoblotting using anti-C-Spc72p antibodies. 6His–C-Spc72p^{231–622} bound to GST–Kar1p (lane 2) was already detectable in the Coomassie Blue stained gel (asterisk). The molecular masses of the standard proteins are indicated in kDa. (E) *SPC72* is a high dosage suppressor of the mating defect of *kar1-1* cells. Cells of *KAR1* (lanes 1 and 2; ESM643-1/2) and *kar1-1* (lanes 3 and 4; ESM614-1/2) carrying either pRS426 (lanes 2 and 4) or *SPC72* on the multi-copy vector pRS426 (pRS426–*SPC72*, lanes 1 and 3) were crossed with wild-type cells (strain JY431). Karyogamy efficiency was determined as described in Materials and methods.

Spc72p-GFP signal (Figure 2A, lower). We established that the karyogamy deficiency and the Spc72p localization defect of *kar1-Δ15* cells were complemented by a plasmid carrying *KAR1* and that the SPB in the *kar1-Δ15* cell body was labelled when the central plaque component Spc42p-GFP (Bullitt *et al.*, 1997) was used as SPB marker (data not shown). Thus, in mating cells the association of Spc72p with the SPB depends on the N-terminal domain of Kar1p.

It has been shown that deletion of the outer plaque component Cnm67p impairs CM organization by the outer plaque. In these cells all CMs originate from the half bridge (Brachat *et al.*, 1998; illustrated in Figure 8C). Since Spc72p is associated with the outer plaque (Knop and Schiebel, 1998; Wigge *et al.*, 1998) and in a Kar1p-dependent manner with the half bridge (Figure 2A and B), Spc72p should not

be associated with the SPB in the *kar1-Δ15 Δcnm67* double mutant and these cells should hardly be viable, because Spc72p fails to bind to the SPB and therefore is unable to fulfil its essential function in our strain background (Chen *et al.*, 1998; Knop and Schiebel, 1998). It is important to note that for unknown reasons *SPC72* is not essential in some other strains (Souès and Adams, 1998).

The viability of cells carrying the *kar1-Δ15 Δcnm67* mutations was investigated using a plasmid shuffle approach (Figure 2C). In agreement with a previous report (Vallen *et al.*, 1992), *kar1-Δ15* cells grew as wild-type [compare sector 2 (*kar1-Δ15*) with 1 (wild-type)]. In contrast, *Δcnm67* cells (sectors 3 and 4) showed a growth defect (Brachat *et al.*, 1998) which was increased further by the *kar1-Δ15* mutation, indicated by the fact that no *Δcnm67 kar1-Δ15* colonies were obtained after 3 days

(sectors 5 and 6). However, rare survivors were observed after 6 days which were tested for the localization of Spc72p (Figure 2D). Whereas Spc72p was associated with nearly all SPBs ($\geq 97\%$, $n = 120$) in wild-type (*KAR1 CNM67*), *kar1-Δ15* (*kar1-Δ15 CNM67*) and $\Delta cnm67$ (*KAR1 Δcnm67*) cells throughout the cell cycle (Figure 2D, compare the anti-Spc98p signals with the anti-Spc72p signals), it was not detectable at the SPB in *kar1-Δ15 Δcnm67* cells. Instead, Spc72p was visualized as a few dots in cells with one DAPI staining region (Figure 2D, upper large budded cell in *kar1-Δ15 Δcnm67*) or in cells

with multiple nuclei (Figure 2D, lower cell in *kar1-Δ15 Δcnm67*) and these dots did not co-localize with the anti-Spc98p signals, representing the SPBs. The anti-Spc72p dots may represent Spc72p polymers, which might form due to the self-assembly properties of Spc72p (Souès and Adams, 1998). Cells of *kar1-Δ15 Δcnm67* were also found to be packed with up to 15 nuclei (Figure 2D, large budded cell on the bottom left), possibly caused by a severe nuclear migration failure due to the lack of CMs. Taken together, the SPB localization of Spc72p in vegetatively growing cells is dependent on a functional outer

plaque and on the N-terminal domain of Kar1p throughout the cell cycle. In contrast, in mating cells the SPB localization of Spc72p is solely dependent on Kar1p.

Our data indicate that Spc72p is associated with the outer plaque and via Kar1p with the half bridge of the SPB (Figure 2D). In *kar1-Δ15* cells Spc72p is probably only associated with the outer plaque. Therefore, the disappearance of the Spc72p signal in *kar1-Δ15* cells after the addition of pheromone suggests that the amount of Spc72p at the outer plaque of the SPB is decreased drastically in response to mating pheromone (Figure 2A and B). However, it is unclear whether Spc72p at the half bridge is regulated by the pheromone pathway and whether Spc72p fluctuates within the SPB in a cell-cycle-dependent manner during vegetative growth. To investigate this, the

fluorescence intensity of 20 SPBs of *SPC72-GFP* cells was determined. We found that the SPB signal of α -factor arrested wild-type cells (relative fluorescence intensity of 6.7 ± 1.7) was higher compared with cells in G_1 (4.7 ± 2.3) or in mitosis (4.7 ± 1.6). This increased Spc72p-GFP signal observed in α -factor arrested cells, in which Spc72p is solely associated with the half bridge via Kar1p (Figure 2A and B), not only indicates that in response to pheromone Spc72p dissociates from the outer plaque but also that more Spc72p binds to the SPB half bridge. In addition, we followed the Spc72p-GFP signal in *kar1-Δ15* cells, in which Spc72p is only associated with the outer plaque. In these cells the Spc72p-GFP signal was lower in G_1 (1.3 ± 0.5) compared with mitosis (4.0 ± 3.0) and no signal above background was observed

Fig. 2. *In vivo* binding of Spc72p to the SPB requires Kar1p and Cnm67p during vegetative growth, but is only dependent on Kar1p in mating cells. (A) The SPB localization of Spc72p during conjugation is dependent on Kar1p. *SPC72-GFP* wild-type cells (ESM617) were marked with Concanavalin A conjugated to Texas Red. The washed cells were then incubated for 1–3 h at 30°C with *SPC72-GFP KAR1* (ESM504) or *SPC72-GFP kar1-Δ15* (YMK232) cells to obtain intermediates of the karyogamy process. Spc72p-GFP in paraformaldehyde-fixed cells was visualized by fluorescence microscopy. DNA was stained with DAPI. Cells were also inspected by phase-contrast microscopy. Two yeast cells are shown whose cell bodies have fused, however, karyogamy has not occurred. Spc72p-GFP is associated with both SPBs when two wild-type cells were crossed (upper panel). Spc72p-GFP was only associated with the SPB of the wild-type cell (marked with concanavalin Texas Red) when a wild-type cell was crossed with a *kar1-Δ15* cell (black arrow). Bar, 3.5 μ m. (B) Binding of Spc72p to the SPB in mating cells is dependent on the Spc72p-binding domain of Kar1p. *SPC72-GFP* wild-type (ESM504) and *SPC72-GFP kar1-Δ15* (YMK232) cells were incubated with or without α -factor for 2 h at 30°C. Cells were inspected by phase-contrast and fluorescence microscopy (Spc72p-GFP). Panels 1–4 display wild-type cells with (3 and 4, black arrows) or without (1 and 2) α -factor. Panels 5–7 show cells of *kar1-Δ15* which were incubated with (black arrow) or without pheromone (red arrow). Cells incubated with and without α -factor were mixed in order to compare the Spc72p-GFP signals. Panels 6 and 7 show different planes of the same cells. The white arrows in 2, 4, 6 and 7 point towards Spc72p-GFP signals. Bar in B5: 3.5 μ m. (C) Deletion of *CNM67* and the *kar1-Δ15* mutation are synthetically lethal. A strain was constructed which was chromosomally deleted in *CNM67* and which carried *CNM67* on the *URA3*-based plasmid pRS316 (ESM431). *KAR1* of ESM431 was replaced by *kar1-Δ15* (YMK207). Growth of cells of ESM431 with the *LEU2*-based plasmid pRS315 (*KAR1 Δcnm67*, sectors 3 and 4) or *CNM67* on pRS315 (*KAR1 CNM67*, sector 1) was tested for 3 days at 30°C on plates containing 5-fluoroorotic acid (5-FOA) which selects against the *URA3*-based plasmid pRS316-*CNM67*. Similarly, cells of YMK207 with pRS315 (*kar1-Δ15 Δcnm67*, sectors 5 and 6) or with *CNM67* on pRS315 (*kar1-Δ15 CNM67*, sector 2) were grown on 5-FOA. The failure of ESM431 (pRS315) cells to grow on 5-FOA (sectors 5 and 6) indicates synthetic lethality of *Δcnm67* and *kar1-Δ15*. (D) Localization of Spc72p with the SPB is dependent on the N-terminal domain of Kar1p and on Cnm67p. Wild-type cells (*KAR1 CNM67*; YPH499) and cells of *Δcnm67* (*KAR1 Δcnm67*; ESM424), *kar1-Δ15* (*kar1-Δ15 CNM67*; YMK179) and *Δcnm67 kar1-Δ15* (ESM658) were investigated by indirect immunofluorescence with anti-Spc98p and anti-Spc72p antibodies. DNA was stained with DAPI. The anti-Spc98p and anti-Spc72p signals were merged. The yellow colour of the merged signals indicates co-localization. Bar, 6.0 μ m.

Fig. 3. The *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ gene fusion functions for *KAR1* and *SPC72*. (A) Schematic diagram of Kar1p, Kar1-1p, Kar1-Δ15p and the *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ protein. Amino acids 106–192 of Kar1p (green) are involved in karyogamy, whereas the C-terminal domain (red and blue) containing the hydrophobic membrane anchor (blue) is essential for SPB duplication and for the localization of Kar1p to the half bridge of the SPB (Vallen *et al.*, 1992). Kar1-1p carries a single point mutation (P150S) that reduces karyogamy efficiency (Conde and Fink, 1976; Vallen *et al.*, 1992). In Kar1-Δ15p amino acids 106–192 of Kar1p are deleted (Vallen *et al.*, 1992). The *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ protein carries amino acids 192–433 of Kar1p, which are essential for SPB duplication (Vallen *et al.*, 1992), but it lacks the entire N-terminus of Kar1p. This domain of Kar1p was substituted by the Tub4p-complex-binding domain of *SPC72*¹⁻²⁷⁶ (yellow; Knop and Schiebel, 1998). (B) The *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ gene fusion functions for *KAR1* and *SPC72*. The *LEU2*-based plasmid pRS315 (sector 1) and *SPC72* (sector 2), *KAR1* (sector 3) or *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ (sector 4) in pRS315 were transformed into cells of strain ESM33 (*Δkar1::HIS3* pRS316-*KAR1*; plate *Δkar1*) and of ESM448 (*Δspc72::kanMX6* pRS316-*SPC72*; plate *Δspc72*). The transformants were tested for growth on 5-FOA plates at 30°C for 3 days. Growth on 5-FOA indicates that the gene in pRS315 is functional for either *KAR1* (plate *Δkar1*) or *SPC72* (plate *Δspc72*). Cells of *Δspc72 Δkar1 SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ (UGY64) with either *KAR1* (plate *Δkar1*, sector 5) or *SPC72* (plate *Δspc72*, sector 5) on pRS316 were also grown of 5-FOA. (C and D) The *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ protein is a SPB component. A logarithmically growing culture of cells carrying *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ but lacking *SPC72* (UGY51) (C) or *SPC72* and *KAR1* (UGY64) (D) were treated with or without α-factor for 2 h at 30°C. The *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ protein and Spc98p were visualized by indirect immunofluorescence using anti-N-Spc72p and anti-98 kDa antibodies (Rout and Kilmartin, 1990), respectively. DNA was stained with DAPI. Cells were also inspected by phase-contrast microscopy. Bars, 2.5 μm.

after α-factor treatment. This result is consistent with the notion that during vegetative growth the amount of Spc72p at the outer plaque decreases in G₁ of the cell cycle. However, this decrease is less pronounced compared with the total dissociation of Spc72p from the outer plaque in response to a pheromone signal.

A *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ hybrid functions for *SPC72* and *KAR1*

Our results suggest that the main function of Kar1p in CM organization is to recruit the Tub4p-complex-binding protein Spc72p to the half bridge. Spc72p may then interact with the Tub4p complex which organizes CMs. These functions of Kar1p and Spc72p are also fulfilled by a *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ fusion protein consisting of the Tub4p-complex-binding site of Spc72p (amino acids 1–276) and amino acids 192–433 of Kar1p that target Kar1p to the half bridge (illustrated in Figure 3A). Since the *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ fusion protein contains the domain of Kar1p required for its essential function in SPB duplication (Vallen *et al.*, 1992), this protein may function not only for Spc72p but also for Kar1p.

A plasmid shuffle experiment revealed that the

*SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ gene fusion expressed from the *KAR1* promoter complemented the essential functions of *KAR1* (Figure 3B; *Δkar1*, sector 4), of *SPC72* (*Δspc72*, sector 4) and of *SPC72* and *KAR1* (*Δkar1* or *Δspc72*, sector 5). For the further analysis, *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ was integrated into a chromosomal location. The *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ protein was located at the SPB in *Δspc72 SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ (Figure 3C, –α-factor) and in *Δspc72 Δkar1 SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ cells (Figure 3D, –α-factor) throughout the cell cycle and in cells exposed to pheromone (Figure 3C and D, +α-factor). Quantitation of the SPB signals revealed that the amount of *SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ at the SPB was approximately similar to Spc72p (data not shown). Furthermore, the analysis of 15 complete serial sections through SPBs of large budded *Δspc72 SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ (Figure 4B and C) or *Δkar1 Δspc72 SPC72*¹⁻²⁷⁶-*KAR1*¹⁹²⁻⁴³³ cells (Figure 4E) did not reveal CMs connected with the outer plaque in any of the sections as was the case in all 10 inspected large budded wild-type (Figure 4A) and *kar1-Δ15* cells (data not shown). Instead, in the mutant cells all CMs originated from the half bridge (Figure 4B, C and E). This analysis shows that CMs are organized differently

in large budded $\Delta spc72$ $SPC72^{1-276}$ – $KAR1^{192-433}$ cells with or without $KAR1$ in comparison with wild-type. In contrast, the organization of CMs was similar in small budded $\Delta spc72$ $SPC72^{1-276}$ – $KAR1^{192-433}$ (Figure 4D) and wild-type cells (data not shown; Byers, 1981). In both cell types the CMs started from the bridge (Figure 4D), which connects the two side-by-side SPBs. Taken together, these data suggest that the $Spc72^{1-276}$ – $Kar1^{192-433}$ protein fulfils the functions of Spc72p and of Kar1p at the half bridge and bridge of the SPB.

CMs at the half bridge can fulfil most of the functions of the CMs of the outer plaque

Cells of $\Delta spc72$ $\Delta kar1$ $SPC72^{1-276}$ – $KAR1^{192-433}$ have no CMs at the outer plaque (Figure 4) and therefore lack the major CM array of wild-type cells. Owing to this unique property, we were able to address whether CMs of the half bridge can fulfil the functions of the outer plaque microtubules. Analysis of the microtubules of vegetatively growing cells with $SPC72^{1-276}$ – $KAR1^{192-433}$, but without $KAR1$, $SPC72$ or $KAR1$ $SPC72$, by indirect immunofluorescence revealed that $\geq 93\%$ of these cells had normal microtubule arrays (Figure 5A, Table I). These cells formed a functional mitotic spindle (white arrows) which was aligned along the mother-to-bud axis by the CMs (yellow arrows). The CMs extended from the SPB to the cell cortex and their appearance was as in wild-type cells. In addition, the chromosomes were segregated in cells with a large bud and the nucleus had migrated into the bud. Phenotypes such as two nuclei in the mother cell body or a misaligned mitotic spindle, which are caused by a malfunction of the CMs (Sullivan and Huffaker, 1992; Eshel *et al.*, 1993; Li *et al.*, 1993; Knop and Schiebel, 1998), were only observed in a few cells (Figure 5A, red arrows; Table I). Similarly, when any of these mutant cells were treated with pheromone most CMs appeared as in wild-type cells (Figure 5B; Table II). A broad bundle of nuclear microtubules (white arrow) and a fainter array of CMs (yellow arrow), separated by the SPB in the nuclear envelope, were aligned along the length axis of the cell. However, some pheromone-arrested mutant cells had abnormal CMs (Table II). In these cells, CMs were either not connected with the SPB (Figure 5B, yellow asterisk), a phenotype which was most prominent in $\Delta kar1$ $SPC72^{1-276}$ – $KAR1^{192-433}$ cells (Table II), or were not aligned along the length axis of the cell (Figure 5B, white and red asterisks).

Defects in CM organization affect the efficiency of karyogamy during conjugation (Rose and Fink, 1987). However, $\Delta spc72$ $SPC72^{1-276}$ – $KAR1^{192-433}$ cells were not karyogamy deficient when crossed to wild-type cells [Figure 5C, bar 1 (wild-type), bar 6 ($\Delta spc72$ $SPC72^{1-276}$ – $KAR1^{192-433}$)] and only a very moderate mating defect was observed by crossing both mutant cell types (Figure 5C, bar 3), which was $\sim 1/40$ of the defect when $kar1$ – $\Delta 15$ cells were crossed with each other (zygote/diploid ratio of 80). Cells of $\Delta spc72$ $\Delta kar1$ $SPC72^{1-276}$ – $KAR1^{192-433}$ (Figure 5C, bars 4 and 7) behaved similar to $\Delta spc72$ $SPC72^{1-276}$ – $KAR1^{192-433}$ cells (bars 3 and 6). Despite the occurrence of free CMs in some pheromone-treated $\Delta kar1$ $SPC72^{1-276}$ – $KAR1^{192-433}$ cells (Table II), these cells had hardly any karyogamy defect either when crossed to wild-type (Figure 5C, bar 5) or to mutant cells

Fig. 4. The CMs of $\Delta spc72$ $SPC72^{1-276}$ – $KAR1^{192-433}$ cells originate at the half bridge of the SPB. (A) SPB of a large budded wild-type cell (YPH499) with CMs attached to the outer plaque. (B) A SPB of a large budded $\Delta spc72$ $SPC72^{1-276}$ – $KAR1^{192-433}$ cell (UGY51) is shown with a central plaque and a half bridge and the CMs originate from the half bridge. (C) Two consecutive sections through one SPB of a $\Delta spc72$ $SPC72^{1-276}$ – $KAR1^{192-433}$ cell with a large bud are shown. The section on the left-hand side shows the half bridge with CMs. The next section reveals the central and outer plaque of the SPB and the outer plaque is not associated with CMs. Other sections through this SPB did not reveal CMs originating from the outer plaque (data not shown). The asterisk in (C) indicates a nuclear pore. (D) A small budded $\Delta spc72$ $SPC72^{1-276}$ – $KAR1^{192-433}$ cell in which the SPB has just duplicated. The two side-by-side SPBs are connected via the bridge, but not the outer plaques, is associated with CMs. (E) Two sections through a SPB of a large budded $\Delta kar1$ $\Delta spc72$ $SPC72^{1-276}$ – $KAR1^{192-433}$ cell (UGY64). The left-hand side shows the central and outer plaque of the SPB and no CMs are associated with the outer plaque. The next section reveals the half bridge which is associated with CMs. Other sections through this SPB did not reveal CMs originating from the outer plaque (data not shown). Abbreviations: B, bridge; CM, cytoplasmic microtubules; CP, central plaque; HB, half bridge; NE, nuclear envelope; NM, nuclear microtubules; OP, outer plaque.

(bar 2). In summary, CMs attached to the half bridge via the $Spc72^{1-276}$ – $Kar1^{192-433}$ protein fulfil most of the functions of the outer plaque microtubules during veget-

Fig. 5. The CMs of $SPC72^{1-276}-KAR1^{192-433}$ cells that originate from the half bridge are functional. (A, B) Wild-type cells (YPH499) and cells of $SPC72^{1-276}-KAR1^{192-433}$ without $SPC72$ (UGY51), $KAR1$ (UGY46) or $SPC72$ and $KAR1$ (UGY64) were grown in YPDA medium at 30°C to a density of 5×10^6 cells/ml. A portion of the cells were used directly for indirect immunofluorescence (A, log cells), whereas the rest was incubated further with α -factor for 2 h at 30°C followed by indirect immunofluorescence with anti-tubulin antibodies (B, α -factor). DNA was stained with DAPI. Cells were inspected by phase-contrast microscopy. (A) The yellow, white and red arrows point towards CMs, the mitotic spindle or cells with a nuclear migration defect, respectively. (B) The nuclear microtubules are marked with a white arrow, whereas the yellow arrows point toward CMs. The yellow, white and red asterisks highlight CMs which are defective. (A) Bar, 5 μ m. (B) Bar, 2.5 μ m. (C) Cells of $\Delta kar1 \Delta spc72$ $SPC72^{1-276}-KAR1^{192-433}$ show only a weak karyogamy defect. Karyogamy efficiency of wild-type and mutant cells was determined as described in Materials and methods. Shown is the mean value of three experiments. Bar 1, wild-type (ESM670) crossed to wild-type (ESM673); bar 2, both cell types (UGY55 and UGY58) were $SPC72^{1-276}-KAR1^{192-433} \Delta kar1$; bar 3, both cell types (UGY56 and UGY59) were $SPC72^{1-276}-KAR1^{192-433} \Delta spc72$; bar 4, both cell types (GPY134 and UGY78) were $SPC72^{1-276}-KAR1^{192-433} \Delta kar1 \Delta spc72$; bar 5, wild-type (ESM673) crossed to $SPC72^{1-276}-KAR1^{192-433} \Delta kar1$ (UGY55); bar 6, wild-type (ESM673) crossed to $SPC72^{1-276}-KAR1^{192-433} \Delta spc72$ (UGY56); bar 7, wild-type (ESM673) crossed to $SPC72^{1-276}-KAR1^{192-433} \Delta kar1 \Delta spc72$ (GPY134).

ative growth. In addition, since the karyogamy defect was small, we conclude that the CMs of $SPC72^{1-276}-KAR1^{192-433}$ cells are also functional during conjugation.

Spc72p, Kar1p and components of the Tub4p complex are required to maintain the CMs at the half bridge

We investigated whether components of the Tub4p complex (Tub4p, Spc98p and Spc97p) and proteins that anchor the Tub4p complex to the half bridge (Kar1p and Spc72p)

are required to maintain the CMs after they have been reorganized in response to pheromone. Conditional lethal $kar1-\Delta 17$, $spc72-7$, $spc97-20$, $spc98-13$ and $tub4-1$ mutant cells were incubated with α -factor at the permissive temperature (23°C), followed by a short incubation at the restrictive temperature (37°C) in the presence of pheromone.

Wild-type cells exposed to pheromone showed a faint bundle of CMs that was directed from the SPB into the mating projection (Figure 6, yellow arrow), whereas the

Table I. Phenotypes of cells expressing the *SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³* gene fusion

<i>SPC72 KAR1</i>	60	11	29	0	0
<i>SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³ Δspc72</i>	63	9	21	4	3
<i>SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³ Δkar1</i>	68	7	22	2	0
<i>SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³ Δspc72 Δkar1</i>	70	9	21	0	0

Cells were grown to early logarithmic phase and prepared for fluorescence microscopy as described in Materials and methods. Approximately 150 cells were counted. The shaded area within the cells indicates the DAPI staining. Values are given in percentages.

Table II. Phenotypes of the pheromone-treated cells expressing the *SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³* fusion gene

<i>SPC72 KAR1</i>	95	5	0	0
<i>SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³ SPC72 KAR1</i>	77	22	1	0
<i>SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³ Δkar1</i>	70	7	6	17
<i>SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³ Δspc72</i>	87	8	3	2
<i>SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³ Δspc72 Δkar1</i>	93	1	1	5

Cultures at a density of 5×10^6 cells/ml were treated with α -factor for 2.3 h at 30°C. Cells were prepared for indirect immunofluorescence with anti-tubulin antibodies as described in Materials and methods. Approximately 150 cells with a mating projection were counted. Values are given in percentages. The dot indicates the SPB. The bar in the nucleus represents the nuclear microtubules. The thin lines denote the position of the CMs.

broader nuclear microtubules (white arrows) were aligned along the length axis of the cell (black arrow) and overlapped with the DAPI staining region. Frequently, we observed a gap in between the two microtubule bundles (pink arrow), reflecting the localization of the SPB. Both microtubule bundles were present in 97% of the wild-type cells when incubated at 37°C.

We observed that >95% of CMs of the pheromone-treated mutant cells appeared normal at the permissive temperature. In contrast, most CMs of *spc72-7* (80%), *spc97-20* (52%) and *spc98-13* (87%) cells were no longer detectable after an incubation at the restrictive temperature, indicating that the encoded proteins have an important role in maintaining the CMs in mating cells (Figure 6). The effect of the *tub4-1* mutation was not as severe, but still 25% of the *tub4-1* cells lacked CMs, which is clearly above the wild-type level of 3%. In cells carrying the *kar1-Δ17* mutation, which causes a temperature-sensitive growth defect (Vallen et al., 1992), detached microtubules were observed in ~15% of the cells (Figure 6, yellow arrow) and most of the remainder showed misaligned or no (5%) CMs. Furthermore, the nuclear microtubules of cells lacking or having detached CMs were not aligned along the length axis of the cell (Figure 6, black arrow).

Fig. 6. Kar1p, Spc72p and components of the Tub4p complex are required to maintain the CMs after exposure of cells to pheromone. Cells of wild-type (YPH499), *spc72-7* (YMK205), *spc97-20* (YMK51.20), *spc98-13* (GPY13-13A), *tub4-1* (ESM218) and *kar1-Δ17* (MS2083) were grown to mid-logarithmic phase in YPD medium at 23°C. α -Factor was added and the cells were incubated for 2.5 h at 23°C until $\geq 95\%$ of the cells showed a mating projection. Cells were resuspended in 37°C pre-warmed YPD medium containing α -factor followed by an incubation for 30 min at 37°C. Cells were analysed by phase-contrast microscopy and indirect immunofluorescence using anti-tubulin antibodies. DNA was stained with DAPI. Bar, 2.5 μ m.

In contrast, the CMs of pheromone-treated $\Delta cnm67$ cells, which lack an outer plaque, were not affected at 37°C (data not shown; Brachat *et al.*, 1998). In summary, Kar1p, Spc72p and components of the Tub4p complex are involved in the maintenance of the CMs in cells treated with mating pheromone.

The Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein is part of a complex containing Kar1p and proteins of the Tub4p complex

To obtain biochemical proof for the involvement of the Tub4p complex in CM organization during mating, we tested whether the Tub4p complex is associated with Spc72p in pheromone-treated cells, as this is the case during vegetative growth (Knop and Schiebel, 1998). Tub4p, Spc98p and Spc72p were co-immunoprecipitated with Spc97p-3HA using anti-HA antibodies (Figure 7A, lane 1). We also noticed that Spc98p and Spc72p were resolved into multiple bands due to the phosphorylation of these proteins (Pereira *et al.*, 1998, G.Pereira, unpublished). This immunoprecipitation was specific, since it was not observed when *SPC97* cells were used (Figure 7A, lane 2). Thus, the Tub4p complex is in association with Spc72p in pheromone-exposed cells.

Attempts to co-immunoprecipitate Kar1p together with either Spc72p-3HA or Spc97p-3HA were not successful (data not shown). We reasoned that the Kar1p-Spc72p interaction may be destabilized as soon as cells are homogenized or during immunoprecipitation. If this is the case, we should be able to co-immunoprecipitate the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein together with the Tub4p complex. Indeed, we found that in extracts from vegetatively growing $\Delta kar1 \Delta spc72$ *SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³-6HA* cells the components of the Tub4p complex (Figure 7B, lane 1) were co-immunoprecipitated with the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³-6HA protein (Figure 7B, anti-Spc72p blot). This precipitation was dependent on the HA-tag on the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein (Figure 7B, lane 2). Thus, the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein is associated with the Tub4p complex.

This result was confirmed using an extract from vegetatively growing *KAR1-3HA $\Delta spc72$ SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³* cells (Figure 7C). The Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein (anti-Spc72p blot), the components of the Tub4p complex and Kar1p-3HA were immunoprecipitated by the anti-HA antibodies (Figure 7C, lane 1) and this was not observed when an extract from *KAR1 $\Delta spc72$ SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³* cells was used (lane 2). Taken together, in *KAR1 $\Delta spc72$ SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³* cells, Kar1p is in a larger complex with the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein and the Tub4p complex. In addition, the co-immunoprecipitation of the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein with the half bridge component Kar1p (Spang *et al.*, 1995) further supports our notion that the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein is associated with the half bridge of the SPB.

Discussion

Spc72p at the half bridge has only a minor role during vegetative growth

The budding yeast SPB organizes two spatially distinct CMs arrays. CMs are attached to the outer plaque of the

Fig. 7. Co-immunoprecipitation of Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein and components of the Tub4p complex together with Kar1p-3HA. (A) Co-immunoprecipitation of Spc72p, Tub4p, Spc97p and Spc98p. *SPC97* cells (GPY64; lane 2) and cells of *SPC97-3HA* (GPY65; lane 1) were treated with α -factor for 2.5 h at 30°C until $\geq 95\%$ of the cells showed a mating projection. Spc97p-3HA was immunoprecipitated using anti-HA antibodies (12CA5). Spc97p, Spc98p, Tub4p and Spc72p in the immunoprecipitate were determined by immunoblotting using affinity-purified antibodies. (B) Co-immunoprecipitation of the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³-6HA protein with Spc97p, Spc98p and Tub4p. Lysates of vegetatively growing $\Delta kar1 \Delta spc72$ *SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³-6HA* (UGY80; lane 1) or $\Delta kar1 \Delta spc72$ *SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³* cells (UGY64; lane 2) were incubated with anti-HA antibodies followed by protein G beads. The Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³-6HA protein (anti-Spc72p blot), Spc97p, Spc98p and Tub4p were detected in the immunoprecipitate using anti-N-Spc72p, anti-Spc97p, anti-Spc98p or anti-Tub4p antibodies. (C) Co-immunoprecipitation of Kar1p-3HA with the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein, Spc97p, Spc98p and Tub4p. Lysates from cells of *KAR1-3HA $\Delta spc72$ SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³* (GPY135; lane 1) or *KAR1 $\Delta spc72$ SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³* (UGY59; lane 2) were incubated with anti-HA antibodies. The indicated proteins were detected in the immunoprecipitate by immunoblotting.

SPB throughout the cell cycle, whereas in G_1 of the cell cycle additional CMs originate from the half bridge of the SPB (Byers and Goetsch, 1975). Recently, we showed that the Tub4p complex is targeted to the outer plaque of the SPB via its binding to Spc72p (Knop and Schiebel, 1998; Figure 8A), providing some molecular understanding for how these microtubules are organized. Here we present evidence that Spc72p is not only associated with the outer plaque, but also interacts with the N-terminal domain of the half bridge component Kar1p (Figures 1 and 2). Therefore, it is possible that during vegetative growth Kar1p targets Spc72p to the half bridge and that Spc72p then recruits the Tub4p complex to this SPB substructure to initiate the formation of microtubules. This model is further supported by the observation that during vegetative growth the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein and the Tub4p complex were co-immunoprecipitated with the half bridge component Kar1p (Figure 7). In addition, while this manuscript was in preparation, Adams and Kilmartin (1999) have shown by immunoelectron microscopy that Spc72p and Tub4p are associated with the SPB half bridge in G_1 cells.

To understand whether the association of Spc72p with the SPB outer plaque and with the half bridge is cell-cycle regulated, we quantitated the Spc72p-GFP signal of SPBs of wild-type and *kar1-Δ15* cells. Cells of *Δkar1-Δ15* proved particularly useful in this study, because Spc72p is only associated with the outer plaque but not with the half bridge of the SPB as this is the case in wild-type cells (Figure 2A and B). We found that the Spc72p-GFP signal of SPBs of *kar1-Δ15* cells decreased by two-thirds when cells progressed from mitosis into G_1 raising the possibility that the association of Spc72p at the outer plaque is to some extent regulated throughout the cell cycle. In contrast, the Spc72p-GFP SPB signal of wild-type cells was the same in G_1 and in mitosis. We propose that in wild-type cells a decrease of Spc72p in G_1 at the outer plaque is balanced by an increase of Spc72p at the half bridge. Such a cell-cycle-dependent fluctuation of Spc72p and Tub4p within the SPB was also most recently suggested based on immunoelectron microscopy (Adams and Kilmartin, 1999). It is possible that Spc72p shows a similar cell-cycle distribution within the SPB as the CMs (Byers and Goetsch, 1975). How the distribution of Spc72p within the SPB is regulated throughout the cell cycle is unclear, but we noted that Spc72p phosphorylation is decreased in G_1 of the cell cycle (G.Pereira, unpublished).

Using the *kar1-Δ15* and *Δcnm67* mutations, which affect the CMs of either the half bridge or the outer plaque (Figure 8B and C), respectively, we addressed whether the two CM arrays have specific functions. Cells of *kar1-Δ15* lack the Spc72p-binding domain of Kar1p (Figure 1) and therefore fail to organize the CMs of the half bridge, a conclusion which was confirmed by electron microscopy (data not shown). Despite this deficiency, no obvious defect has been observed in *kar1-Δ15* cells during vegetative growth (Vallen *et al.*, 1992; Figure 2C and D). However, a role of the CMs of the half bridge became apparent in *Δcnm67* cells, whose viability depends on the CMs organized by Kar1p (Figure 2C). Taken together, these results suggest that the CMs of the half bridge have only a minor function during vegetative growth.

Fig. 8. Model for the function of Spc72p and Kar1p in CM organization. (A) Schematic diagram of the outer/central plaque of a wild-type SPB. The nuclear inner plaque is not illustrated. The central plaque is embedded within the nuclear envelope. The central/outer plaques contain layers of Spc42p, Cnm67p and Nud1p/Spc94p (Wigge *et al.*, 1998; Elliott *et al.*, 1999). The N-terminal domain of Spc72p binds at the outer plaque to Spc97p and Spc98p of the Tub4p complex (Rout and Kilmartin, 1990; Geissler *et al.*, 1996; Knop *et al.*, 1997; Knop and Schiebel, 1997, 1998; Pereira *et al.*, 1998). Kar1p and Cdc31p are components of the SPB half bridge that interact (Spang *et al.*, 1993, 1995; Vallen *et al.*, 1994). For further details see text. (B) CM organization of *kar1-Δ15* cells. In pheromone exposed *kar1-Δ15* cells Spc72p is no longer detectable at the SPB suggesting that the association of Spc72p with the SPB is entirely dependent on the Spc72p-binding domain of Kar1p. (C) In *Δcnm67* cells the CMs are organized by the half bridge. Cells deleted in *CNM67* do not have an outer plaque and are therefore defective in CM organization by the outer plaque. All CMs are organized by the half bridge (Brachet *et al.*, 1998). (D) The *SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³* gene fusion functions for *KAR1* and *SPC72*. The C-terminal domain of the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein fulfils the essential function of Kar1p during SPB duplication, whereas its N-terminus targets the Tub4p complex to the SPB. In the absence of *SPC72* all CMs are organized from the half bridge.

Importantly, this does not mean that the CMs of the half bridge cannot fulfil the functions of the CMs of the outer plaque. Instead, we could clearly demonstrate that the CMs of *Δspc72 SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³* or *Δkar1 Δspc72 SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³* cells, which originate exclusively

from the half bridge or bridge of the SPB (Figure 4B–E), were of normal appearance during vegetative growth (Figure 5A). Consequently, functional deficiencies, such as a nuclear migration defect, were only found in a small percentage of cells (Table I). This result also suggests that the cell-cycle-dependent distribution of the CMs within the SPB is not essential for many CM-directed processes.

The function of Kar1p during conjugation is to target Spc72p to the half bridge of the SPB

KAR1 has been identified as one of the first genes involved in karyogamy (Conde and Fink, 1976). In this study we provide the first molecular understanding of the role of Kar1p during conjugation. We show that Kar1p recruits Spc72p to the half bridge in response to a pheromone signal, and this is the major function of Kar1p during karyogamy. Our conclusion is based on the following findings. First, Spc72p interacts with a region in Kar1p which has been implicated as the domain of Kar1p that functions in karyogamy (Vallen *et al.*, 1992; Figure 1A). Secondly, the interaction of Spc72p with Kar1p is impaired by mutations in Kar1p, such as Kar1-1p (Figure 1B and C), which specifically affect karyogamy. Thirdly, the mating defect of *kar1-1* cells was in part suppressed by the overexpression of *SPC72* (Figure 1E). Finally, a hybrid protein in which the N-terminal of Kar1p has been replaced by the Tub4p-complex-binding domain of Spc72p (Figure 8D; Knop and Schiebel, 1998), and thereby mimics the function of the Kar1p–Spc72p complex, fulfilled the role of Kar1p and Spc72p in mating (Figures 3 and 4; Table II). However, it is important to note that we do not exclude the possibility that Kar1p has additional, probably minor roles during karyogamy. This is suggested by the CM defects which occur in some $\Delta kar1$ *SPC72*^{1–276}–*KAR1*^{192–433} cells when arrested with α -factor (Table II). Kar1p may recruit other proteins to the site of microtubule organization which then stabilize the attachment of the CMs to the half bridge.

The addition of pheromone to yeast cells results in the rearrangement of the CMs (Byers and Goetsch, 1975; Vallen *et al.*, 1992) and of Spc72p (Figure 2A and B) such that their association with the SPB is entirely dependent on the half bridge component Kar1p. This raises the question of whether the pheromone-induced relocalization of Spc72p is a direct consequence of the pheromone response pathway (Leberer *et al.*, 1997) or caused indirectly by the Far1p-induced cell-cycle arrest of mating yeast cells in G₁ of the cell cycle (Chang and Herskowitz, 1990). Our results suggest that Spc72p has the capability to bind to the outer plaque of the SPB in vegetatively growing G₁ cells (Figure 2B and D). This contrasts with the situation of pheromone-arrested cells in which Spc72p's association with the SPB is solely dependent on the half bridge component Kar1p (Figure 2A and B). In addition, we observed that the Spc72p-GFP signal at the SPB of α -factor-arrested wild-type cells was higher compared with vegetatively growing G₁ cells, suggesting that the amount of Spc72p at the half bridge increases when cells are exposed to mating pheromone. Thus, in response to a pheromone signal more Spc72p binds to the half bridge and significantly less Spc72p is observed at the outer plaque. Importantly, the extent of these changes exceeds the cell-cycle-dependent fluctuations of Spc72p within the

SPB. Considering that Spc72p interacts with the Tub4p complex (Knop and Schiebel, 1998) which is involved in microtubule organization (Pereira and Schiebel, 1997), the relocalization of Spc72p within the SPB is possibly the 'landmark' event in pheromone-induced reorganization of the CMs.

Other proteins that are directed to the SPB by a mating pheromone signal are the kinesin-related protein Kar3p and its associated protein Cik1p (Meluh and Rose, 1990; Page *et al.*, 1994). Similar to Spc72p, the Kar3p/Cik1p complex changes its localization in response to pheromone. The Kar3p/Cik1p complex is found along the mitotic spindle in vegetatively growing cells (Page *et al.*, 1994; Manning *et al.*, 1999), whereas it is associated with the SPB in a Kar1p-dependent manner and along CMs in pheromone-treated cells (Meluh and Rose, 1990; Page and Snyder, 1992). It is known that transcription of *KAR3* and *CIK1* is increased in mating cells (Meluh and Rose, 1990; Page and Snyder, 1992; Kurihara *et al.*, 1996), a mode of regulation that has been excluded for *SPC72* (G.Pereira, unpublished results). Whether this increased expression of *KAR3* and *CIK1* is sufficient to target the Kar3p/Cik1p complex to the SPB in a Kar1p-dependent manner is unclear. It is possible that for the Kar3p/Cik1p complex, and also for Spc72p, other regulatory mechanisms play a role. Spc72p, the outer-plaque-binding protein of Spc72p or Kar1p may be regulated by phosphorylation in response to mating pheromone as this is the case for Far1p and the transcription factor Ste12p (Chang and Herskowitz, 1992; Elion *et al.*, 1993).

Spc72p was found to be associated with the Tub4p complex in mating cells (Figure 7A). In addition, using temperature-sensitive mutants, we obtained evidence that Kar1p, Spc72p and the components of the Tub4p complex are still required to maintain the half bridge microtubules after they have been formed in response to a mating pheromone signal (Figure 6). These observations raise the possibilities that either the CMs are stable after their formation and remain attached to the half bridge via the Kar1p, Spc72p and the Tub4p complex or that the CMs are unstable and constantly reformed at the half bridge by a *de novo* nucleation process. In the first case, the CMs detach from the SPB half bridge in temperature-sensitive *spc72*, *spc97*, *spc98* and *tub4* mutants and the released CMs depolymerize. In the second case, the continuous reformation of the CMs is impaired in the temperature-sensitive mutants. We consider the second possibility less likely, since CMs grow and shorten primarily at the shmoo tip in mating cells, and not at the SPB (Maddox *et al.*, 1999). In addition, the free CMs observed in *kar1- Δ 17* cells (Figure 6) are most easily explained by the first model, assuming that in *kar1- Δ 17* cells the CMs detach together with the Tub4p/Spc72p complex, which then stabilizes these microtubules. Thus, our data support a model in which in response to a pheromone signal Spc72p is released from the outer plaque and more Spc72p is targeted to the half bridge via Kar1p. Spc72p then recruits the Tub4p complex to this SPB substructure to induce the formation of CMs. After their formation, these CMs remain attached to the half bridge via the binding of the Tub4p complex to Spc72p and Spc72p to Kar1p (Figure 8A).

Microtubule reorganization processes

We have shown that budding yeast has at least two so-called γ -tubulin complex-binding proteins (GTBPs), Spc72p and Spc110p, which target the cytoplasmically assembled Tub4p complex (Pereira *et al.*, 1998) to the sites of microtubule organization within the SPB. Spc110p is the GTBP of the inner plaque, whereas Spc72p has been identified as the GTBP of the outer plaque (Knop and Schiebel, 1997, 1998). The results described in this paper extend the model of how GTBPs work. The same GTBP may be associated with multiple centrosomal sub-structures or with different microtubule-organizing centres (MTOCs) within one cell. GTBPs may change their distribution within the centrosome in response to external and internal signals by binding to different GTBP-binding

proteins such as Kar1p. By this means the microtubules may gain specific properties which enable them to fulfil specialized functions. Our data further suggest that γ -tubulin complexes, GTBPs and GTBP-binding proteins not only have a function in microtubule formation, but also have additional roles in the attachment of microtubules to MTOCs.

Materials and methods**General yeast methods**

Standard protocols were used for growth and transformation of yeast cells. Yeast strains were grown in yeast extract, peptone, dextrose (YPD) medium containing 100 mg/ml adenine (YPDA) or in synthetic complete (SC) medium (Sherman, 1991). Yeast strains used in this study were

Table III. Yeast strains and plasmids used in this study

Name	Genotype	Source or reference
Yeast strains		
ESM33	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δkar1::HIS3 pSM30</i>	Spang <i>et al.</i> (1995)
ESM218	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tub4-1</i>	Spang <i>et al.</i> (1996)
ESM356-1	<i>MATa ura3-53 trp1Δ63 his3Δ200 leu2Δ1</i>	Knop <i>et al.</i> (1998)
ESM357-9	<i>MATa ura3-53 trp1Δ63 his3Δ200 leu2Δ1</i>	Knop <i>et al.</i> (1998)
ESM424	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δcnm67::kanMX4</i>	this study
ESM431	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δcnm67::kanMX4 pSM431</i>	this study
ESM448	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc72::kanMX4 pSM447</i>	Knop <i>et al.</i> (1998)
ESM479	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 SPC72-3HA-kanMX6</i>	Knop <i>et al.</i> (1998)
ESM504	<i>MATa ura3-53 leu2Δ1 trp1Δ63 his3Δ200 SPC72-GFP-kanMX4</i>	this study
ESM612-1	YMK207 pRS315	this study
ESM612-2	YMK207 pSM449	this study
ESM614-1	EY93 pSM458	this study
ESM614-2	EY93 pRS426	this study
ESM617	<i>MATa ura3-53 leu2Δ1 trp1Δ63 his3Δ200 SPC72-GFP-kanMX4</i>	this study
ESM643-1	JY425 pRS426	this study
ESM643-2	JY425 pSM458	this study
ESM657-1	ESM431 pRS315	this study
ESM657-2	ESM431 pSM449	this study
ESM658	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δcnm67::kanMX4 kar1-Δ15</i>	this study
ESM670	<i>MATa ura3-52::pRS306 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1</i>	this study
ESM673	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63::pRS304 his3Δ200 leu2Δ1</i>	this study
EY93	<i>MATa his4-34 leu2-3,112 ura3-52 kar1-1</i>	Cold Spring Harbor Yeast Course
GPY13-13A	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc98-13</i>	this study
GPY64	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δsst1::HisG</i>	this study
GPY65	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 SPC97-3HA-kanMX6 Δsst1::HisG</i>	this study
GPY134	<i>MATa ura3-52::pRS306 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::pUG11 Δspc72::kanMX4 Δkar1::HIS3</i>	this study
GPY135	UGY64 pBM12	this study
MS2083	<i>MATa trp1 leu2 ura3-52 kar1-Δ17</i>	Cold Spring Harbor Yeast Course
SGY37	<i>MATa ura3-52::URA3-lexA-op-LacZ trp1 his3 leu2</i>	Geissler <i>et al.</i> (1996)
UGY46	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::pUG11 Δkar1::HIS3</i>	this study
UGY51	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::pUG11 Δspc72::kanMX4</i>	this study
UGY55	<i>MATa ura3-52::pRS306 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::pUG11 Δkar1::HIS3</i>	this study
UGY56	<i>MATa ura3-52::pRS306 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::pUG11 Δspc72::kanMX4</i>	this study
UGY58	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63::pRS304 his3Δ200 leu2Δ1::pUG11 Δkar1::HIS3</i>	this study
UGY59	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63::pRS304 his3Δ200 leu2Δ1::pUG11 Δspc72::kanMX4</i>	this study
UGY64	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::pUG11 Δspc72::kanMX4 Δkar1::HIS3</i>	this study
UGY78	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63::pRS304 his3Δ200 leu2Δ1::pUG11 Δspc72::kanMX4 Δkar1::HIS3</i>	this study
UGY80	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1::SPC72¹⁻²⁷⁶-KAR1¹⁹²⁻⁴³³-6HA-kITRP1::LEU2 Δspc72::kanMX4 Δkar1::HIS3</i>	this study
JY425	<i>MATa his4-34 leu2-3,112 ura3-52</i>	Cold Spring Harbor Yeast Course
JY431	<i>MATa his4-34 trp1Δ1 ura3-52</i>	Cold Spring Harbor Yeast Course
YMK51.20	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc97-20</i>	Knop <i>et al.</i> (1997)
YMK179	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 kar1-Δ15</i>	this study
YMK205	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc72-7</i>	Knop <i>et al.</i> (1998)
YMK207	<i>MATa ura3-53 leu2Δ1 trp1Δ63 his3Δ200 kar1-Δ15 Δcnm67::kanMX4 pSM431</i>	this study
YMK232	<i>MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200 kar1-Δ15 SPC72-GFP-kanMX4</i>	this study
YPH499	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1</i>	Sikorski and Hieter (1989)
YPH500	<i>MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1</i>	Sikorski and Hieter (1989)

derivatives of S288c except for strains EY93, JY425 and JY431 used in the *kar1-1* suppression experiment. Strains and plasmids are listed in Table III. The *kar1-Δ15* mutation was introduced by 'pop-in pop-out' of the integration vector pMK211. The *kar1-1* allele was cloned by gap repair using strain EY93. All strains constructed during the course of this study were tested for the presence or absence of Cnm67p, Spc72p, Kar1p and the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein by immunoblotting with affinity-purified antibodies and by colony PCR with specific primers. MATa yeast cells were synchronized with 1 μg/ml (*Δsst1* cells) or 10 μg/ml (*SST1* cells) of synthetic α-factor until ≥95% of the cells showed a mating projection.

Two-hybrid interactions

Two-hybrid interactions were determined with derivatives of pACT2 (Gyuris *et al.*, 1993) and pEG202 (Dufree *et al.*, 1993) which were transformed into the reporter strain SGY37 (Geissler *et al.*, 1998). β-galactosidase activity was determined either by a plate assay with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as a substrate (Geissler *et al.*, 1998) or in Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol, pH 7.0) with *o*-nitrophenyl-β-D-galactopyranoside (ONPG). In the latter case, the activity was normalized to the optical density of the culture (Kaiser *et al.*, 1994).

Immunological techniques, fluorescence and electron microscopy

The relative fluorescence intensity raised by Spc72p-GFP at the SPB was determined (NIH image program) as the difference between the maximal fluorescence of the SPB and the background fluorescence measured at the immediate proximity of the SPB. These measurements were taken in the linear range of our detection system. Immunofluorescence and electron microscopy of yeast cells was performed using

published protocols (Kilmartin and Adams, 1984; Byers and Goetsch, 1991). Microtubules were detected with the mouse monoclonal antibody (mAb) WA3 (Spang *et al.*, 1996). Affinity-purified rabbit anti-Spc72p antibodies were used to detect Spc72p (Knop and Schiebel, 1998). Spc98p was visualized by indirect immunofluorescence with a pool of mouse anti-90 kDa mAb (Rout and Kilmartin, 1990). Secondary antibodies were from Jackson Research Laboratories. Kar1p-3HA or Spc97p-3HA were immunoprecipitated with anti-HA antibodies bound to protein G-Sepharose (Knop and Schiebel, 1998). Anti-HA antibodies (12CA5) were from Hiss Diagnostics. Affinity-purified rabbit anti-Kar1p, anti-N-Spc72p, anti-Spc97p, anti-Spc98p and anti-Tub4p antibodies were used to test the immunoprecipitates (Spang *et al.*, 1995, 1996; Geissler *et al.*, 1996; Knop *et al.*, 1997; Knop and Schiebel, 1998). The anti-N-Spc72p antibodies also recognized the Spc72¹⁻²⁷⁶-Kar1¹⁹²⁻⁴³³ protein. Antibodies directed against the C-terminal domain of Spc72p were affinity-purified using a histidine-tagged Spc72p²³¹⁻⁶²².

In vitro binding of C-Spc72p to GST-Kar1p

Spc72²³¹⁻⁶²² was cloned into the *E. coli* expression vector pET28c (pMK297). *KAR1*, *kar1-1* and *kar1-Δ15* were ligated into pGEX-5X-1. pET28c and pGEX-5X-1 derivatives were transformed into *E. coli* BL21 DE3 (Studier and Moffat, 1986). Recombinant proteins were expressed upon the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 30°C. GST fusion proteins were bound to glutathione-Sepharose (Pharmacia) as recommended by the manufacturer. An *E. coli* extract containing 6His-Spc72²³¹⁻⁶²² was incubated with the glutathione beads for 1 h at 4°C in phosphate-buffered saline (PBS) containing 0.1% Triton-X100. The resin was washed once with PBS, 0.1% Triton-X100, followed by two washes with PBS. Bound proteins were eluted with SDS-sample buffer and analysed by Coomassie Blue staining and immunoblotting using anti-C-Spc72p antibodies.

Table III. Continued

Name	Genotype	Source or reference
Plasmids		
pACT2	2 μm, <i>LEU2</i> -based vector carrying the <i>GAL4</i> activator domain	Dufree <i>et al.</i> (1993)
pBM12	pRS315 containing <i>KAR1</i> -3HA	Spang <i>et al.</i> (1995)
pEG202	2 μm, <i>HIS3</i> -based vector carrying the <i>lexA</i> DNA-binding domain	Gyuris <i>et al.</i> (1993)
pET28c	<i>E. coli</i> expression vector containing 6His under control of the T7 promoter	Novagen
pGEX-5X-1	<i>E. coli</i> expression vector containing GST under control of the <i>lacZ</i> promoter	Pharmacia
pGP65 ^a	pEG202 containing <i>SPC72</i> ¹⁻⁶²²	Knop <i>et al.</i> (1998)
pGP66	pEG202 containing <i>SPC72</i> ¹⁻²⁶⁷	Knop <i>et al.</i> (1998)
pGP67	pEG202 containing <i>SPC72</i> ²³¹⁻⁶²²	Knop <i>et al.</i> (1998)
pMK198	pACT2 containing <i>KAR1</i> ¹⁻⁴⁰⁸	this study
pMK207	pACT2 containing <i>KAR1</i> ¹¹⁶⁻²³⁶	this study
pMK208	pACT2 containing <i>KAR1</i> ²³⁵⁻²⁷⁴	this study
pMK209	pACT2 containing <i>KAR1</i> ¹¹⁶⁻²⁷⁴	this study
pMK211	pRS306 containing <i>kar1-Δ15</i>	this study
pMK297	pET28c containing <i>SPC72</i> ²³¹⁻⁶²²	this study
pRS304	<i>TRP1</i> -based yeast integration vector	Sikorski and Hieter (1989)
pRS305	<i>LEU2</i> -based yeast integration vector	Sikorski and Hieter (1989)
pRS306	<i>URA3</i> -based yeast integration vector	Sikorski and Hieter (1989)
pRS315	<i>CEN6</i> , <i>LEU2</i> -based yeast- <i>E. coli</i> shuttle vector	Sikorski and Hieter (1989)
pRS316	<i>CEN6</i> , <i>URA3</i> -based yeast- <i>E. coli</i> shuttle vector	Sikorski and Hieter (1989)
pRS414	<i>CEN6</i> , <i>TRP1</i> -based yeast- <i>E. coli</i> shuttle vector	Sikorski and Hieter (1989)
pRS426	2 μm, <i>URA3</i> -based yeast- <i>E. coli</i> shuttle vector	Christianson <i>et al.</i> (1992)
pSM30	pRS316 containing <i>KAR1</i>	Spang <i>et al.</i> (1995)
pSM95	pGEX-5X-1 containing <i>KAR1</i> ¹⁻³⁸⁶	Spang <i>et al.</i> (1995)
pSM119	pRS315 containing <i>KAR1</i>	Spang <i>et al.</i> (1995)
pSM412	pRS315 containing <i>SPC72</i>	Knop <i>et al.</i> (1998)
pSM431	pRS316 containing <i>CNM67</i>	this study
pSM447	pRS316 containing <i>SPC72</i>	this study
pSM449	pRS315 containing <i>CNM67</i>	this study
pSM458	pRS426 containing <i>SPC72</i>	this study
pSM628	pRS315 containing <i>kar1-1</i>	this study
pSM630	pACT2 containing <i>kar1-Δ15</i> ¹⁻⁴⁰⁸	this study
pSM631	pACT2 containing <i>kar1-1</i> ¹⁻⁴⁰⁸	this study
pSM632	pGEX-5X-1 containing <i>kar1-Δ15</i> ¹⁻⁴⁰⁸	this study
pSM633	pGEX-5X-1 containing <i>kar1-1</i> ¹⁻⁴⁰⁸	this study
pUG9	pRS315 containing <i>SPC72</i> ¹⁻²⁷⁶ - <i>KAR1</i> ¹⁹²⁻⁴³³	this study
pUG11	pRS305 containing <i>SPC72</i> ¹⁻²⁷⁶ - <i>KAR1</i> ¹⁹²⁻⁴³³	this study

^a*SPC72*¹⁻⁶²² indicates that codons 1-622 of *SPC72* have been cloned into plasmid pEG202.

Quantitative mating

Yeast strains with the genotypes *MATa Ura⁺ trp1* (ESM670, UGY55, UGY56, GPY134) or *MATa ura3-52 Trp⁺* (ESM673, UGY58, UGY59, UGY78) were grown at 30°C in SC medium lacking uracil or tryptophan, respectively, to a density of 10⁷ cells/ml. Cells (3 × 10⁶ cells) of both mating types were mixed and filtered onto a nitrocellulose membrane. The membrane was placed onto a YPD plate which was incubated for 4 h at 30°C. Cells were washed with PBS and dilutions were plated onto YPD plates (number of viable cells) and SC plates lacking tryptophan and uracil (number of diploid cells). Both plates were incubated for 3 days at 30°C. The percentage of diploid cells was determined. The cells in PBS were also inspected using light microscopy to determine the percentage of zygotes in the suspension. In case of the *kar1-1* suppression analysis by *SPC72*, *MATa leu2-3,112 Trp⁺* cells (ESM643-1/2; ESM614-1/2) with plasmids pRS426 or pRS426-*SPC72* were grown at 30°C in SC medium lacking uracil to a density of 10⁷ cells/ml. JY431 (*MATa trp1Δ1 Leu⁺*) cells were grown in YPD medium at 30°C. After mating, cells were plated onto YPD plates and SC plates without leucine and tryptophan to determine the percentage of diploid cells.

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