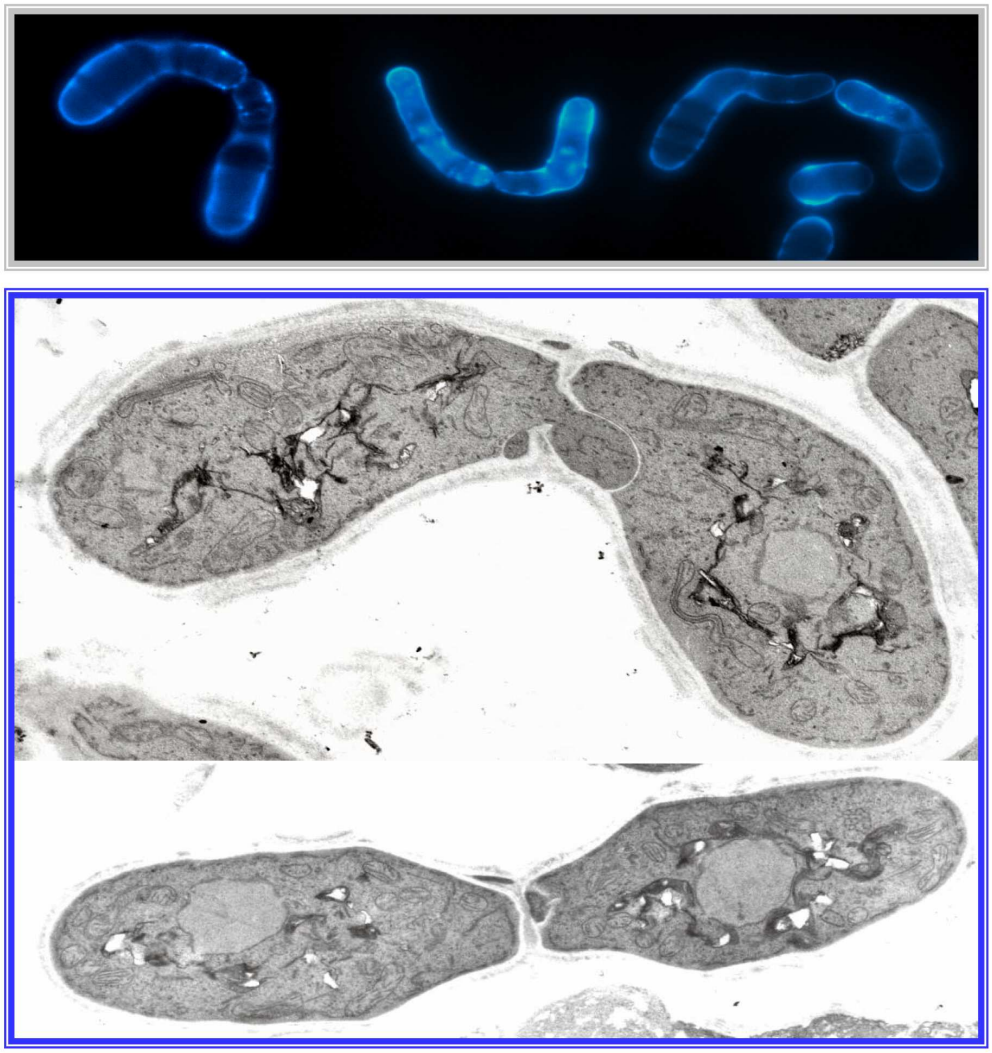

Cover illustration: the tetraspan protein Dni1p from *Schizosaccharomyces pombe* is required for membrane organization and cell wall remodeling during mating. Calcofluor staining (upper panel) and electron microscopy (lower panel) showed that cells do not fuse in *dni1Δ* zygotes.

156x165mm (300 x 300 DPI)

The tetraspan protein Dni1p is required for correct membrane organization and cell wall remodelling during mating in *Schizosaccharomyces pombe*

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Running title: The tetraspan Dni1p is required for cell fusion

Key words: yeast, cell wall, mating, cell fusion, membrane microdomains, tetraspan proteins

Summary

In fungi, success of mating requires that both cells agglutinate, modify their extracellular envelopes, and fuse their plasma membranes and nuclei to produce a zygote. Here we studied the role of the *Schizosaccharomyces pombe* Dni1 protein in the cell fusion step of mating. Dni1p is a tetraspan protein bearing a conserved cystein motif similar to that present in fungal claudin-related proteins. Dni1p expression is induced during mating and Dni1p concentrates as discrete patches at the cell-cell contact area and along the mating bridge. Proper Dni1p localization depends on Fus1p, actin, and integrity of lipid rafts. In *dni1Δ* mutants, cell differentiation and agglutination are as efficient as in the WT strain, but cell fusion is significantly reduced at temperatures above 25ºC. We found that the defect in cell fusion was not associated with an altered cytoskeleton, with an abnormal distribution of Fus1p, or with a defect in calcium accumulation, but with a severe disorganization of the plasma membrane and cell wall at the area of cell-cell contact. These results show that Dni1p plays a relevant role in coordinating membrane organization and cell wall remodelling during mating, a function that has not been described for other proteins in the fission yeast.

Introduction

Conjugation is a complex event directed to ensure the transfer of genetic material that requires that both parental cells differentiate and fuse, giving rise to a diploid zygote. Studies undertaken in animals have allowed some aspects of cellular fusion to be elucidated (Wassarman, 1999, Kaji & Kudo, 2004, Stein *et al.*, 2004), although in most cases the genes involved in the process have not yet been identified. In the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, a considerable body of information has been obtained regarding pheromone-induced signalling, sexual differentiation, meiosis, and spore formation (Marsh & Rose, 1997, Yamamoto *et al.*, 1997, Davey, 1998, Arcangioli & Thon, 2004, Nielsen, 2004, Shimoda & Nakamura, 2004, Yamamoto, 2004, Nakamura *et al.*, 2008). Nevertheless, very little is known about the mechanism leading to the fusion step of mating in these model organisms, in particular in the fission yeast.

In *S. pombe*, heterothallic strains belong to one of two specialized cell types: M (h^- cells) or P (h^+ cells). Additionally, there is a homothallic strain (h^{90}) that changes its mating type during proliferation (see Arcangioli & Thon, 2004). Mixed populations of h^- and h^+ cells can proliferate actively in rich medium without conjugating. In the absence of nitrogen, the level of cAMP decreases in the cell, which results in the dissociation of a heterotrimeric G protein and the *ste11*$^+$-mediated expression of many genes involved in sexual development (Yamamoto *et al.*, 1997, Davey, 1998, Nielsen, 2004). Then, cells produce mating pheromones that bind to specific receptors present in the membrane of the cells belonging to the opposite mating type and initiate the mating process by differentiating into specialized cells called shmoos. Shmoos are G1-arrested

cells that grow in a polarized way to facilitate the contact with a shmoo from the other mating type (Nielsen, 2004). Then, the Mam3p and Map4p agglutinins (Yamamoto *et al.*, 1997, Mata & Bahler, 2006, Sharifmoghadam *et al.*, 2006) facilitate and strengthen the union between the cells (Calleja & Johnson, 1971, Calleja, 1987).

When the union between the parental cells is stable, the cell walls that separate them degrade and the plasma membranes fuse, allowing diffusion of the cytoplasmic contents between both cells (Calleja *et al.*, 1977a). This step of the mating process has been subjected to detailed microscopic analyses (Streiblová & Wolf, 1975, Calleja *et al.*, 1977a, Calleja *et al.*, 1977b) but has not been characterized at the molecular level. It is known that a functional cytoskeleton is required for this process, since mutations in *cdc3*$^{+}$ or *cdc8*$^{+}$ (coding for profilin and tropomyosin, respectively), and treatment with microtubule-destabilizing agents lead to a defect in cell fusion (Petersen *et al.*, 1998a, Petersen *et al.*, 1998c, Kurahashi *et al.*, 2002). Cell fusion also requires Fus1p and Cfr1p. *fus1Δ* and *cfr1Δ* mutants have no apparent defects during vegetative growth, but they show a defect in cell fusion. Fus1p is a formin-homologue that localizes to the tip of the shmoo and is required for the organization and stabilization of F-actin at the projection tip (Petersen *et al.*, 1995, Petersen *et al.*, 1998c, Petersen *et al.*, 1998b). Cfr1p is a Golgi protein that regulates mating through a Fus1p-independent pathway (Cartagena-Lirola *et al.*, 2006). Once the cells fuse, karyogamy takes place, producing a diploid zygote. In *S. pombe,* diploids are unstable so they undergo meiosis immediately unless they are kept in rich medium. When meiosis is complete, four haploid spores develop (Yamamoto *et al.*, 1997, Davey, 1998, Nielsen, 2004).

Since the cells are only able to initiate mating in a nitrogen-scarce medium, systematic studies have been undertaken using this condition to identify the genes that participate in this process (Mata *et al.*, 2002, Mata & Bahler, 2006). In one of these studies, the genes that were induced in minimal medium without nitrogen were grouped in clusters according to their time of expression (Mata *et al.*, 2002). *fus1*$^{+}$ belongs to the cluster denoted as "Genes induced in response to nitrogen starvation (delayed)", which includes several genes involved in the production of pheromones and their receptors. To gain information about the cell fusion step of mating, we are studying some of the genes included in that cluster. In a previous work we characterized the SPBC21D10.06C ORF, which turned out to code for the *h*$^{+}$-specific agglutinin Map4p (Sharifmoghadam *et al.*, 2006). Here, we describe the characterization of *dni1*$^{+}$ (from *d*elayed minus-*n*itrogen *i*nduction, corresponding to the SPAC31G5.07 ORF).

According to computer-assisted predictions, Dni1p is a tetraspan (a protein with four transmembrane domains). There are different families of tetraspans that share a common topology and structure, with some residues conserved at specific positions of the extracellular loops, although there are also atypical members of each family. Dni1p is most similar to some fungal members of the claudin-stargazing family of proteins, which include the Sur7-related and the Fig1-related proteins (Muller *et al.*, 2003, Walther *et al.*, 2006, Zhang *et al.*, 2006, Alvarez *et al.*, 2008, Grossmann *et al.*, 2008. See figures 1 and S1). The Sur7-related proteins have been implicated in protein turnover because they are associated with eisosomes, which are endocytic sites at the plasma membrane, and with the MCC domains (ergosterol-rich Membrane Compartments of Can1), which are sites where the proteins are protected from endocytosis (Walther *et al.*, 2006, Alvarez *et al.*, 2008, Grossmann *et al.*, 2008). Fig1p-

related proteins participate in the Low-Affinity Ca^{++} influx System (LACS) and are required for proper cell fusion during mating in *S. cerevisiae* (Erdman *et al.*, 1998, Muller *et al.*, 2003, Aguilar *et al.*, 2007), and for polarized growth in *C. albicans* (Brand *et al.*, 2007). Dni1p shares 21% identity and 45% similarity with Fig1p. Since LACS activation only occurs in nitrogen-rich media (Muller *et al.*, 2001), and since *S. pombe* cells do not mate under these conditions (Yamamoto *et al.*, 1997, Davey, 1998, Nielsen, 2004), we wanted to study whether Dni1p played any role in calcium uptake and/or cell fusion during mating. We found that Dni1p localized as a discrete patch to the tip of the shmoo. *dni1Δ* null mutants showed a temperature-dependent defect in cell fusion that is not aggravated by the deletion of *dni2*$^+$ (a second *S. pombe* Fig1p homologue). Genetic and microscopic analyses revealed that Dni1p and Fus1p are functionally related, Fus1p being necessary for the correct localization of Dni1p to the tip of the shmoo. We also found that Dni1p was required for the correct organization of the plasma membrane and the coordination between membrane- and cell wall-remodelling at the area of cell-cell contact.

Results

Dni1p belongs to the fungal family of claudin-stargazing proteins

According to the data existing in the databases, the SPAC31G5.07 ORF is induced during the initial moments of mating and codes for a protein that shares sequence similarity with the *Saccharomyces cerevisiae* Fig1p. A closest sequence analysis allowed us to find out that the SPBC4.01 ORF codes for a second *FIG1* homologue that is also expressed in response to nitrogen deprivation (Mata *et al.*, 2002). We have termed these genes *dni1*$^+$ and *dni2*$^+$, respectively. Figure 1 shows a sequence alignment between *S. cerevisiae* Fig1p and Sur7p, *S. pombe* Dni1p and Dni2p, *C. albicans* Sur7p,

and human Claudin1. The alignment shows the conserved aminoacids in the Cys-containing GΦΦGxC(8-20 aa)C motif, where Φ= Y,F,L or M. This motif is characteristic of the Fig1-family proteins and is related to the WxxW/YxxC(7-10 aa)C motif present in the Sur7-family proteins and to the GLWxxC(8-10 aa)C motif present in claudins (Zhang *et al.*, 2006, Alvarez *et al.*, 2008). The alignment indicates that Dni1p and Dni2p belong to the family of fungal claudin-stargazing proteins.

Dni1p levels increase in response to nitrogen starvation and Dni1p localizes to discrete patches at the tip of shmoos

The results obtained from genome-wide analyses showed that *dni1*$^{+}$ was up-regulated in response to pheromones or nitrogen deprivation (Mata *et al.*, 2002, Mata & Bahler, 2006). In order to confirm that Dni1p was involved in mating, we analyzed the level of this protein during this process by Western blot. We found that the amount of Dni1p (pointed by an arrow in figure 2 A) increased after 2 hours of incubation in the absence of nitrogen, reaching a peak after 4-6 hours of incubation. Thereafter, the Dni1p signal decreased and new bands that might correspond to degradation products and the GFP alone could be observed (asterisks in figure 2 A). Probably, these bands were produced because the Dni1 portion of Dni1-GFP was degraded after the initial steps of mating releasing the GFP portion. The maximum levels of Dni1p were reached when cells were initiating mating, and before the sporulation efficiency was significant. These results suggested that Dni1p might have a function in mating.

To gain further information about the role of Dni1p in the mating process, we observed cells carrying Dni1-GFP under the fluorescence microscope. We failed to observe any fluorescent signal in the cells during vegetative growth. When we treated *h*$^{-}$ *cyr1Δ*

sxa2Δ cells (which are responsive to pheromones in nitrogen-containing medium and hypersensitive to P factor; Stern & Nurse, 1997) carrying the GFP-fused Dni1 protein with the h^+ pheromone P factor, we found that most Dni1p was localized to the tip of the shmoos (figure 2 B), showing that intercellular contact was not required for the induction of Dni1p. When h^{90} cells were induced to mate, Dni1p was observed as a discrete patch at the area of cell-cell contact in the prezygotes (figure 2 C i), although it was occasionally possible to observe some fluorescent dots in the cell body (figure 2 C ii), or some uniform fluorescence spreading along the plasma membrane (see arrow in figure 2 C iii). Soon after cell fusion had started, the Dni1-GFP signal was observed as discrete dots that accumulated along the mating bridge of the zygotes (figure 2 D). When cell fusion was complete, the protein was observed at the vacuoles, suggesting that it might have undergone some posttraductional modification that rendered it unstable (Figure 2 E). These results were in agreement with the data obtained by Western blotting and suggested that Dni1p might have a specific role in the initial steps of mating.

To determine whether the Dni1 protein was expressed in both mating types, we performed mating assays between h^+ or h^- cells that carried the Dni1-GFP protein and untagged cells of the opposite mating type. We found that Dni1p localized to the cell-cell contact area when it was expressed in cells from either mating type (not shown).

Dni1p localization to the tip of shmoos requires Fus1p, actin, and lipid rafts

We next wanted to study the requirements for Dni1p localization. First, we studied the relationship between *dni1*$^+$ and other genes involved in cell fusion. We analyzed the localization of Dni1p in *cfr1Δ*, *cdc8-F41*, and *fus1Δ* mutants under mating conditions.

As shown in figure 3 A, the localization of Dni1p in the control strain and in the *cfr1Δ* and the *cdc8-F41* mutants was similar. In the *fus1Δ* mutant, we observed that Dni1p exhibited a polarized localization, although the protein was spread along the mating projection instead of being concentrated in a discrete patch at the shmoo tip. In contrast, Fus1p was properly localized in a *dni1Δ* mutant (figure 3 B), suggesting that Dni1p could act downstream from Fus1p.

Since Fus1p is required for the organization of actin patches at the tip of the shmoos (Petersen *et al.*, 1995, Petersen *et al.*, 1998c, Petersen *et al.*, 1998b), we decided to investigate whether the localization of Dni1p depended on actin. To address this question, we treated *cyr1Δ sxa2Δ* shmoos carrying the GFP-fused Dni1 protein with the actin-depolymerizing drug latrunculin A. After this treatment, Dni1p was polarized at the tip of the shmoos but did not concentrate in a patch (figure 3 C), a result that showed that actin was required for the proper localization of Dni1p.

Since Dni1p is a transmembrane protein, we wished to gain information about the influence of membrane organization in its localization. To address this issue, we treated shmoos carrying GFP-tagged Bgs4p (an integral membrane protein) or Dni1p with filipin, an antibiotic that binds to sterols (Drabikowski *et al.*, 1973) and that disrupts lipid rafts when the cells are incubated in its presence for long periods of time (Takeo, 1985, Wachtler *et al.*, 2003). We observed that Bgs4p lost its localization to the projection of the shmoos when the lipid rafts had been disrupted by incubation in the presence of filipin for one hour (figure 3 D, left panels), in agreement with previous results (Wachtler *et al.*, 2003). Similarly, Dni1p was not observed as a neat patch at the tip of the shmoos when the cells had undergone the same treatment (figure 3 D, right

panels). A similar result was obtained when the shmoos were incubated in the presence of the sterol-binding drug methyl-β-cyclodextrin for 1 hour (not shown), showing that the integrity of lipid rafts was required for the proper localization of Dni1p.

dni1^{+} *deletion leads to a defect in cell fusion*

In order to investigate the role of Dni1p, we analyzed the phenotype of a *dni1Δ* mutant and found that this strain showed no obvious phenotype during vegetative growth (data not shown). Next, we performed several experiments in order to know whether Dni1p played any role in mating and to pinpoint the step at which this protein might exert its function. We found that *dni1Δ* cells were able to produce both mating pheromones, and that shmoos were produced in response to P factor with the same efficiency and kinetics in the *cyr1Δ sxaΔ* control cells and in the *cyr1Δ sxaΔ dni1Δ* mutant (data not shown). We next wondered if the *dni1Δ* cells were able to agglutinate in response to nitrogen deprivation, and we found that the agglutination index was the same for the h^{90} *dni1Δ* mutant and for the h^{90} control strain, and that the Map4p agglutinin localized properly in the *dni1Δ* cells (not shown).

When we analyzed the mating process in the h^{90} and the h^{90} *dni1Δ* cells on solid EMM medium, we found that at the time at which the h^{90} strain had sporulated the *dni1Δ* mutant had produced zygotes with long mating bridges, but mature *asci* were scarce (see figure 4 A). In quantitative analyses, we found that the efficiency of mating initiation (This parameter, measured as the number of zygotes plus *asci* with respect to the number of zygotes, *asci*, and cells, reflected the number of cells that were able to differentiate and establish a stable cell-cell contact) was similar in the h^{90} and the h^{90} *dni1Δ* strains (27 and 26%, respectively, n=500). However when we quantified the

sporulation efficiency (This parameter, measured as the number of *asci* with respect to the number of *asci* plus zygotes, reflected the number of zygotes in which cell fusion, meiosis, and spore development had proceeded properly), we found that in the wild-type (WT) strain 83% of the zygotes had produced *asci* after 36 hours of incubation at 32ºC while in the *dni1Δ* mutant strain this value was 6%.

We produced a *dni1Δ/dni1Δ* diploid strain and we found that it sporulated as efficiently as a control diploid strain (not shown). Since in *S. pombe* meiosis proceeds after karyogamy and zygotes give rise to *asci* immediately, this result suggested that the defect in sporulation exhibited by the h^{90} *dni1Δ* mutant was a not a defect in meiosis or spore formation but the consequence of a defect in cell fusion. In order to confirm this hypothesis, we stained the cells with DAPI (for nuclear staining) and Calcofluor (for cell wall staining). As shown in figure 4 B, at the time at which in the control strain the nuclei from both parental cells had fused, in the mutant strain nuclei were still apart from each other. Additionally, cell wall material could be observed between the cells. Concanavalin A staining confirmed that the cell wall was not digested at the cell-cell contact area (figure 4 C). Finally, we introduced the GFP under the control of the $map4^{+}$ promoter into the h^{90} or h^{90} *dni1Δ* strains so that the fluorescent protein was only expressed in the h^{+} cells. We observed that in the WT strain the GFP was present throughout the cytoplasm of the zygotes (figure 4 D), while in the *dni1Δ* mutant the fluorescent signal was only observed on one side of the zygote body of most zygotes (figure 4 D). In the *S. cerevisiae prm1Δ* and *fig1Δ* mutants, which are defective in membrane fusion, it is possible to observe intercellular bubbles in which the fluorescence corresponding to the soluble GFP invades the cell body of one of the mating partners (Heiman & Walter, 2000, Aguilar *et al.*, 2007). We observed this kind

of structure (see arrow in figure 4 D) in less than 10% of the *dni1Δ* zygotes. All these results showed that Dni1p was involved in the cell fusion process and suggested that the lack of fusion was due to a defect in cell wall digestion. Thus, the defect in sporulation observed in the mating mixtures involving the *dni1Δ* mutant was a consequence of a defect in cell fusion. This result would allow sporulation efficiency to be used as an indirect measurement of cell fusion efficiency in the experiments described below.

With a view to analyzing whether the Dni1p function was mating-type associated, we produced h^+ and h^- *dni1Δ* haploid cells and performed unilateral and bilateral crosses. We found that in all crosses cells were able to initiate mating and produce zygotes with similar efficiency (figure 4 E). Regarding the sporulation efficiency, this was 76% for the h^- WT x h^+ WT, 61% for the h^- WT x h^+ *dni1Δ*, 43% for the h^- *dni1Δ* x h^+ WT, and 10% for the h^- *dni1Δ* x h^+ *dni1Δ* crosses (figure 4 E), showing that the presence of Dni1p in one of the mating cells was sufficient to support cell fusion with reasonable efficiency.

This result allowed us to use a genetic approach to confirm the relationship between *fus1*$^+$ and *dni1*$^+$. We compared the sporulation efficiency in bilateral WT x WT, *dni1Δ* x *dni1Δ*, and *fus1Δ* x *fus1Δ* crosses with that obtained in unilateral *dni1Δ* x WT, WT x *fus1Δ* and *dni1Δ* x *fus1Δ* crosses. If both genes acted at different points of the same pathway, the result of the unilateral *dni1Δ* x *fus1Δ* cross would be similar to that of the bilateral *fus1Δ* x *fus1Δ* cross. By contrast, if *fus1*$^+$ and *dni1*$^+$ were acting in parallel pathways, the result of the *dni1Δ* x *fus1Δ* cross would be similar to that of the unilateral *dni1Δ* x WT or WT X *fus1Δ* crosses. As shown in figure 4 F, sporulation efficiency in the unilateral *dni1Δ* x *fus1Δ* cross was similar to that obtained in the bilateral *fus1Δ* x

fus1Δ cross, strongly suggesting that *dni1*$^{+}$ and *fus1*$^{+}$ might act in the same pathway. As a control, the result of a cross involving *fus1Δ* and *cfr1Δ*, which act in different pathways (Cartagena-Lirola *et al.*, 2006), was included.

Membrane fusion and organization, and cell wall remodelling are defective in dni1Δ mutants

All the above results showed that Dni1p was essential for the cell fusion step of mating and pointed to a role of this protein in cell wall digestion or remodelling. In order to gain information about this role, we analyzed *h*^{90} and *h*^{90} *dni1Δ* cells that had been induced to mate at 32ºC, using transmission electron microscopy (figure 5). We observed that in the control strain some membranous structures accumulated at the cell-cell contact area when the cell wall between the parental cells was still present (figure 5 A, i). At later stages, the cell wall could not be observed at the fusion area and the membranes from both cells were apposed (figure 5 A, ii). At the final stage of cell fusion, no membrane or cell wall separated the parental cells (figure 5 A, iii). In the *dni1Δ* mutant, several abnormalities were observed (figure 5 B). An abnormal accumulation of membrane material apposed to the cell wall was present in some cells before cell wall digestion had started (figure 5 B, i). In some cases, the cell wall was partially digested but abnormal membranous structures were observed between the parental cells (figures 5 B, ii, iii, and iv). It was sometimes possible to observe intercellular membrane bubbles that invaded one of the mating cells (figures 5 B, iii, iv,) or intracellular membrane bubbles that grew into one of the mating partners when the cell wall delimiting both cells had not been digested (figures 5 B, v and vi). In some cases, the abnormal membranous structures seemed to contain cell wall material (figures 5 B, ii, iii, iv, and v). At later stages, it was possible to observe that cell wall material

had been synthesized around the membrane bubbles (figure 5 B, vii), and that some cytoplasmic blebs had been trapped by cell wall material (figures 5 B, vii, viii, and ix). These results revealed that in the absence of Dni1p the plasma membrane and cell wall were severely disorganized in the mating cells, and that Dni1p was required for membrane organization and cell wall remodelling during mating.

In order to determine whether Dni1p was a scaffold for the localization of enzymes required for the synthesis and/or degradation of the cell wall at the mating projection, we analyzed the localization of the Bgs1p, Bgs3p, and Bgs4p glucan synthases, and that of the Agn1p and Eng1p glucanases during mating in the h^{90} and the h^{90} *dni1Δ* cells. We did not observe Eng1p in the zygotes from either strain, which is in agreement with the reported downregulation of the expression of this gene at the time of cell fusion (Mata *et al.*, 2002). In contrast, Bgs1p, Bgs3p, Bgs4p, and Agn1p were observed at the cell-cell contact area in both strains (figure S2 and results not shown). We also found that Cdc42p, a GTPase involved in polarity and cell wall synthesis, was localized to the cell-cell contact area in the WT and the *dni1Δ* mutant (figure S2 and results not shown). These results showed that Dni1p is not a general scaffold for the localization of enzymes required for cell wall remodelling.

Dni1p function is more relevant at temperatures above 25ºC

During the development of these studies, we observed that the strength of the cell fusion defect in the *dni1Δ* cells depended on the temperature. To further study this, we quantified the sporulation efficiency in h^{90}, h^{90} *dni1Δ*, and h^{90} *fus1Δ* cells that had been induced to mate at different temperatures (20ºC to 37ºC; see figure 6 A). We found that in the WT strain sporulation was reduced at temperatures above 35ºC, and that the

fus1Δ strain exhibited a severe sporulation defect (less than 1% of the zygotes produced *asci*) at all the temperatures tested. In the *dni1Δ* strain, sporulation was efficient at 20ºC, reduced at 25ºC (50% with respect to the WT), and severely reduced at higher temperatures. Analysis of the sporulation process in *dni1*$^{+}$/*dni1*$^{+}$ and in *dni1Δ/dni1Δ* diploids confirmed that meiosis and spore development proceeded normally in the absence of Dni1p at all temperatures tested (see figure 6 B). Finally, observation of zygotes in which the GFP was expressed under the control of the *map4*$^{+}$ promoter confirmed that cell fusion was indeed more sensitive to temperature in the *dni1Δ* mutant than in the WT strain (not shown). These results showed that Dni1p was dispensable for cell fusion at 20ºC. In order to determine whether Dni2p could undertake the function of Dni1p at low temperatures, *h*^{90}, *h*^{90} *dni1Δ*, *h*^{90} *dni2Δ,* and *h*^{90} *dni1Δ dni2Δ* strains were induced to mate at different temperatures. We found that at 32ºC the *dni1Δ*, the *dni2Δ*, and the *dni1Δ dni2Δ* strains exhibited a similar defect in the efficiency of sporulation, and that at 20ºC sporulation was as efficient in the three mutant strains as in the *h*^{90} control strain (figure 6 C). The absence of intercellular diffusion of a soluble GFP confirmed that the defect in sporulation observed in the *h*^{90} *dni2Δ* and *h*^{90} *dni1Δ dni2Δ* strains was due to a defect in cell fusion (figure 6 D). These results showed that Dni1p and Dni2p cannot substitute each other and suggested that these proteins act together in a fine-tuned mechanism directed to ensuring proper membrane organization and cell wall remodelling during cell fusion at higher temperatures, at which membranes are more fluid and biochemical reactions proceed faster.

In S. pombe, calcium is not accumulated in response to pheromones and the external calcium concentration does not affect cell survival upon sexual differentiation

Since Dni1p shares significant similarity with the *S. cerevisiae* Fig1 protein, which is involved in calcium uptake and cell fusion (Erdman *et al.*, 1998, Muller *et al.*, 2003, Aguilar *et al.*, 2007), we wondered whether Dni1p was involved in calcium uptake during mating. To answer this question calcium accumulation analyses were performed by incubating *cyr1Δ sxa2Δ* and *cyr1Δ sxa2Δ dni1Δ* cells with $^{45}Ca^{++}$ in the presence or absence of the h^{+} pheromone P factor (see Experimental Procedures). As a control, we used an *ehs1Δ* strain (Carnero *et al.*, 2000), which lacks a Ca^{++}-permeable channel (Carnero *et al.*, 2000, Tasaka *et al.*, 2000). The experiment was performed by incubating the cells in medium with or without calcium for different times at 32ºC. As shown in figure 7 A, after four hours of incubation the cells accumulated more $^{45}Ca^{++}$ in the calcium-devoid medium than in the calcium-containing medium, the opposite result from that obtained for different strains in *S. cerevisiae* (Iida *et al.*, 1990). As expected, the *ehs1Δ* strain did not accumulate calcium in any condition tested (figure 7 A). In the WT strain, cells accumulated more calcium when they were treated with pheromone than when they were not, although the difference between both conditions was not as dramatic as that described for *S. cerevisiae* (Iida *et al.*, 1990). Surprisingly, the behaviour of the *dni1Δ* strain was similar to that of the control strain, it being able to accumulate calcium in the presence and the absence of P factor (figure 7 A). Similar results were obtained after one hour and after six hours of incubation (not shown).

It has been described that *S. cerevisiae* cells die when they differentiate into shmoos either in a calcium-deficient medium or in cells lacking the High-Affinity Ca^{++} influx System (HACS) channel Mid1p (Iida *et al.*, 1990, Iida *et al.*, 1994). In order to determine the effect of the external calcium concentration on the viability of *S. pombe* cells treated with pheromone, we exposed *cyr1Δ sxa2Δ* and *cyr1Δ sxa2Δ dni1Δ* cells to

P factor in EMM, EMM-Ca, and EMM-Ca supplemented with 10 mM BAPTA (a calcium-binding agent) or with 100 mM calcium. Samples were taken every hour over six hours and used for microscopic observation and for viability determination by staining the cells with methylene blue and by quantifying the colony-forming units (CFU). We found that cells from both strains produced shmoos with the same efficiency and kinetics, and that more than 90% of these shmoos were viable in all conditions tested (not shown). We also used the methylene blue-staining method to determine zygote lysis in h^{90} and h^{90} *dni1Δ* strains that had been induced to mate in EMM, EMM-Ca, and EMM-Ca supplemented with 10 mM EGTA (a calcium-chelating agent). We found that in both strains lysis was about 10% in all conditions tested. All these results indicated that external calcium was not required for maintaining the viability of *S. pombe* cells in response to pheromone.

Finally, we analyzed the effect of the external calcium concentration on cell fusion. To do so, the sporulation efficiency of an h^{90} *dni1Δ* strain was compared to that of the h^{90} control strain. We found that calcium depletion did not reduce the sporulation efficiency in either the h^{90} or the h^{90} *dni1Δ* strains. Additionally, the sporulation of *dni1*$^{+}$/*dni1*$^{+}$ and *dni1Δ*/*dni1Δ* diploid strains was efficient on EMM, EMM-Ca, and EMM-Ca plates supplemented with 10 mM of EGTA or 100mM calcium (not shown). These results show that the external calcium concentration doest not have a significant influence in the cell fusion process in *S. pombe*. Surprisingly, the addition of 100 mM calcium, but not of 100 mM magnesium, partially rescued the defect in sporulation of the *dni1Δ* mutants (figure 7 B). The same phenomenon has been observed for the *fig1Δ* mutant. It is possible that such high calcium concentrations could induce some cell response that had an indirect effect in cell fusion.

Discussion

In this work, we have investigated the function of Dni1p. *dni1*$^+$ expression is induced in response to nitrogen starvation (Mata *et al.*, 2002), suggesting that Dni1p could play some role in mating. In agreement with this notion we found that this protein accumulated during the initial steps of mating; Dni1p localized to the tip of the shmoos and the mating bridge, and *dni1Δ* cells were defective in cell fusion. We performed several experiments to determine the nature of the mating defect in this strain. It has been described that a functional cytoskeleton is required for cell fusion in *S. pombe* (Petersen *et al.*, 1998a, Petersen *et al.*, 1998c, Kurahashi *et al.*, 2002). We found a genetic interaction between *dni1*$^+$ and *fus1*$^+$, a gene coding for a formin homologue that is required for the organization of actin patches during mating (Petersen *et al.*, 1998c). Immunolocalization analyses showed that microtubules and actin patches were normally distributed in *dni1Δ* zygotes (not shown), and i*n vivo* observation of a *dni1Δ* mutant carrying a GFP-tagged Crn1 protein (Figure S3, Coronin, an actin patch-associated protein; Pelham & Chang, 2001) confirmed the latter result. Additionally, Fus1p was properly localized in the *dni1Δ* mutant (figure 3). All these results indicated that Dni1p does not play a role in organizing the cytoskeleton during mating. Dni1p was not properly localized in *a fus1Δ* mutant (figure 3). Given the general role of formins and the actin cytoskeleton in delivering the majority of membrane proteins via appropriately polarized vesicle trafficking, this result could be interpreted as a dependence of Dni1p localization on actin organization and cell polarity. In fact, actin depolimerization also resulted in Dni1p delocalization (figure 3), in support of this idea. A multicopy plasmid carrying *dni1*$^+$ did not alleviate the mating defect of a *fus1-B20* strain carrying a point mutation in *fus1*$^+$ (Petersen *et al.*, 1995; not shown), which suggested that either Dni1p

could act downstream of Fus1p or that in order to perform its activity Dni1p requires other protein(s) that depend on Fus1p. We found that actin was required for Dni1p localization but, surprisingly, a mutation in tropomyosin (*cdc8-F41* strain; Kurahashi *et al.*, 2002) did not affect that localization. It has been shown that Cdc8p is indispensable for cell fusion during conjugation in fission yeast and it has been speculated that tropomyosin might organize a small F-actin-containing organelle at the cell fusion site (Kurahashi *et al.*, 2002). It is possible that the *cdc8-F41* point mutation might only affect the function of some of proteins localized in that organelle or that this organelle does not contain Dni1p.

Electron microscopy shed light on the function of Dni1p. In the absence of this protein, there was a severe disorganization of the plasma membrane and the cell wall at the cell-cell contact area. Some cells exhibited abnormal membranous structures at the fusion region. We also observed intercellular bubbles and cytoplasmic blebs embedded in the cell wall. This phenotype was not a non-specific consequence of the lack of cell fusion, since in a *cfr1Δ* mutant electron microscopy revealed that the cell wall remained undigested between the parental cells, but intercellular bubbles and blebs were not observed (Cartagena-Lirola *et al.*, 2006). This heterogeneous phenotype could be explained if Dni1p were a regulator of several proteins required for different processes, or if the function of Dni1p were to ensure the coordination between different steps of cell fusion and some of these steps could occasionally proceed in the absence of this protein. The fact that Dni1p function was found to be dispensable at low temperatures, at which biological processes proceed slowly, points in the latter direction. Additionally, we found that the Bgs1p, Bgs3p, Bgs4p glucan synthases and the Agn1p glucanase localized to the mating projections in a *dni1Δ* mutant (figure S2), suggesting

that although Dni1p could be required for the localization of some protein involved in cell wall remodelling, it is not a general scaffold for the localization of the enzymes required for this process.

Dni1p is similar to the *S. cerevisiae* calcium uptake regulator Fig1p. We therefore investigated whether Dni1p and Fig1p were homologues, and we found that there exist a number of similarities and differences between these proteins and their functionalities. When we analyzed the relationship between mating, calcium uptake, and Dni1p we found that calcium accumulation and cell survival, upon exposure of cells to mating pheromones in the absence of calcium, were similar in the WT and *dni1Δ* strains. This is in agreement with the fact that Fig1p is a component of the LACS system (Muller *et al.*, 2003), which mediates calcium uptake in the presence of nitrogen, a condition that inhibits mating in *S. pombe* (Yamamoto *et al.*, 1997, Davey, 1998, Nielsen, 2004). These results suggested that the main function of Dni1p in cell fusion is not to regulate a calcium influx in response to pheromones and that Fig1p and Dni1p are not functional homologues. However, whereas it has been described that in *S. cerevisiae* a strong intracellular calcium accumulation takes place in response to pheromones, and that this calcium accumulation is essential for cell survival upon shmoo formation (Iida *et al.*, 1990, Iida *et al.*, 1994), we failed to detect a strong accumulation of calcium or reduced viability in a calcium-devoid medium in response to pheromones in a *S. pombe* WT strain (figure 7). Additionally, we found that the absence of external calcium did not influence cell fusion during mating in a WT *S. pombe* strain (figure 7 B). It therefore seems that the effect of the external calcium concentration has a different impact on the sexual development on both organisms, a result that would account for the different functionality of Fig1p and Dni1p in regard to calcium uptake/accumulation.

Although Fig1p function was initially associated to the regulation of a calcium-influx system during mating (Muller *et al.*, 2003), it was later described that this protein is also required for the coordination between cell wall remodelling and membrane fusion during mating (Aguilar *et al.*, 2007). Additionally, in *Candida albicans*, Fig1p is involved in calcium influx and polarized growth (Brand *et al.*, 2007, Brand *et al.*, 2009). It is not known whether the calcium influx activity of Fig1p affects cell polarization in *S. cerevisiae* and in *C. albicans*, or whether these are separate functions of the proteins. Additionally, it is still unknown how other proteins work with Fig1p to accomplish certain aspects of polarization, calcium influx, and cell-cell fusion, and how these processes might be interrelated. Thus, it is possible that that Dni1p and Fig1p might function in a related way and that the coordination between cell wall remodelling and membrane fusion, the function that we have proposed for Dni1p and that has also been suggested for Fig1p (Aguilar *et al.*, 2007), is the function most conserved along the evolution of these proteins. Accordingly, Dni1p either represents an out-group of this family of proteins or it is a true homologue of this family but its particular functionality reflects the differences between the budding and fission yeast lineages and their requirements for mating. The evolutionary divergence of these proteins and their partners probably accounts for the fact that Fig1p, expressed under the control of the *dni1*$^{+}$ promoter and terminator sequences in *S. pombe*, localized along the shmoo projections but was unable to complement the mating defect of a *dni1Δ* strain (results not shown). Proteins similar to Dni1p and Fig1p are present in the genomes of other yeasts and fungi. Determining the function of these proteins should help to understand the main role of this family of proteins.

In *S. cerevisiae*, genetic and cytological analyses of mutants defective in mating has led to the establishment of several parallel subpathways that affect different steps of cell-cell fusion (cell signalling, polarity, cell wall remodelling and plasma membrane fusion; Gammie *et al.*, 1998, Ydenberg & Rose, 2008). In *S. pombe*, only two pathways required for cell fusion during mating have been reported; one of them includes the formin-homologue *fus1*$^{+}$ and is involved in actin organization and polarity (Petersen *et al.*, 1995, Petersen *et al.*, 1998b). The other pathway is defined by the Golgi protein Cfr1p, which might be involved in the delivery of a cell wall-degrading enzyme (Cartagena-Lirola *et al.*, 2006). *dni1Δ* mutants exhibit aberrant cell wall and membrane structures at the cell-cell contact area. Some of these structures are reminiscent of those observed in *S. cerevisiae prm1Δ*, *fig1Δ*, *prm1Δ fig1Δ*, and *prm1Δ kex2Δ* mutants (Heiman & Walter, 2000, Aguilar *et al.*, 2007, Heiman *et al.*, 2007), suggesting that *S. pombe* Dni1p might share a function with Fig1p, Prm1p and/or a Kex2-proccessed protein in cell wall remodelling and plasma membrane fusion, defining a new cell fusion subpathway in the fission yeast. In *S. pombe,* the protein encoded by the SPBC4.01 ORF exhibits sequence similarity to Dni1p and Fig1p (figure S1). We analyzed whether this protein (Dni2p) might have a redundant role with Dni1p at low temperatures and we found that the single *dni2Δ* and double *dni1Δ dni2Δ* mutants have a temperature sensitive mating defect equivalent to that of *dni1Δ* mutants. Importantly, the phenotype of the double mutant was no more severe than that of either single mutant. This rules out the hypothesis that the Dni1 and Dni2 proteins functionally substitute for each other, and suggests rather that they have separate essential roles in the same fusion subpathway.

Tetraspan proteins participate in different cellular processes and are sometimes associated with some membrane subdomains (Gonzalez-Mariscal *et al.*, 2003, Stipp *et al.*, 2003, Tarrant *et al.*, 2003, Yunta & Lazo, 2003, Hemler, 2005). Dni1p is a tetraspan similar to the Fig1 and the Sur7 families of claudin-related proteins (Figure 1. Muller *et al.*, 2003, Walther *et al.*, 2006, Zhang *et al.*, 2006, Alvarez *et al.*, 2008, Grossmann *et al.*, 2008). Claudins are the most important components of the tight junctions, where they establish a barrier that controls the flow of molecules in the intercellular space and block the movement of integral membrane proteins. In *C. albicans*, deletion of *SUR7* leads to several phenotypes, including defective endocytosis and an abnormal synthesis of cell wall material that grows into the cytoplasm (Alvarez *et al.*, 2008). Endocytosis of the Map3 pheromone receptor and uptake of FM4-64 seems to take place normally in a *dni1Δ* mutant (figure S4), but an abnormal growth of the cell wall into the cytoplasm was observed in this strain during mating, suggesting that CaSur7p and Dni1p could share some function. Dni1p was observed as a discrete patch, whose integrity required lipid rafts, at the cell fusion area (figure 3). Thus, Dni1p could define a specialized membrane microdomain that would ensure an adequate spatio-temporal regulation of the membrane organization, cell wall remodelling, and cell-cell communication during cell fusion. In *S. cerevisiae,* it has been shown that the composition and distribution of membrane subdomains play a relevant role in mating (Bagnat & Simons, 2002, Proszynski *et al.*, 2006, Jin *et al.*, 2008). The situation is probably similar in *S. pombe*. Finally, it is noteworthy that tetraspanins (a family of tetraspan proteins that cluster in characteristic membrane subdomains; Yunta & Lazo, 2003, Hemler, 2005) play an essential role in sperm-egg fertilization in mammals (for a review see Sutovsky, 2009) and that tetraspan proteins (Fig1p, Prm1p and Dni1p) are required for correct membrane reorganization during mating in yeasts (this work; Heiman & Walter, 2000, Jin *et al.*,

2004, Aguilar *et al.*, 2007, Heiman *et al.*, 2007). A detailed analysis of the role of Dni2p and Prm1p in *S. pombe*, and their relationship with Dni1p, should help to provide information about the relevance of fungal tetraspan proteins in mating.

Experimental procedures

Strains and growth conditions

All general growth conditions and yeast manipulations have been described previously (Moreno *et al.*, 1991; http://www.biotwiki.org/bin/view/Pombe/NurseLabManual). *S. pombe* strains are derivatives of the 972 h^- and 975 h^+ WTs and were grown in YES or EMM. EMM-Ca was prepared by replacing calcium pantothenate by sodium pantothenate. EMM-N was EMM without ammonium chloride. BAPTA and EGTA were from SIGMA.

Mating analysis

Pheromone production was assessed as described previously (Egel *et al.*, 1994). Shmoo formation was induced in *cyr1Δ sxa2Δ* strains by adding synthetically synthesized P factor (Isogen; final concentration of 1.5 μg/ml). *cyr1Δ sxa2Δ* cells are sensitive to low concentrations of pheromones in nitrogen-containing media because they lack the serine carboxypeptidase *sxa2*$^+$, which degrades P factor, and the adenylate cyclase *cyr1*$^+$ (Stern & Nurse, 1997). Shmoos were treated with 100 μM, final concentration, of Latrunculin A (SIGMA) for 10 minutes. Agglutination tests were carried out in EMM-N as described previously (Sharifmoghadam & Valdivieso, 2008). Cells were induced to mate on EMM or EMM-Ca plates at different temperatures. Mating efficiency represented the number of zygotes plus *asci* with respect to the total cell number (zygotes plus *asci* plus vegetative cells), and sporulation efficiency was the number of

asci with respect to the number of *asci* plus zygotes, as described in (Arellano *et al.*, 2000).

Molecular and genetic manipulations

The *dni1*$^+$ ORF and 1kb-DNA fragments corresponding to the 5' and 3' non-coding regions were amplified by PCR, cloned into the KS+ vector (Stratagene), and sequenced. A *dni1Δ* deletion cassette, in which the complete *dni1*$^+$ ORF had been substituted by the KanMX6 gene that confers resistance to geneticin (Bähler *et al.*, 1998), was used to transform the strains of interest. Correct integration was always assessed by PCR. Site-directed mutagenesis was used to introduce a *Not*I restriction site immediately upstream from the stop codon. The GFP was cloned as *Not*I/*Not*I DNA fragment. The GFP-tagged *dni1*$^+$ allele, under the control of its own promoter, was integrated at the *leu1*$^+$ *locus*. A soluble GFP was cloned, as an *Nde*I/*Not*I DNA fragment, into an integrative plasmid that carried the *map4*$^+$ promoter and terminator sequences and that had been digested with those restriction enzymes. The *S. cerevisiae FIG1* gene was PCR-amplified, sequenced, and cloned as a *Sma*I/*Not*I DNA fragment into an integrative plasmid, which carried the *dni1*$^+$ promoter and terminator sequences and which had been digested with those restriction enzymes. The GFP was cloned at the *Not*I site of the latter plasmid. A *dni2::ura4*$^+$ deletion cassette was constructed by cloning 1-Kb DNA fragments corresponding to the *dni2*$^+$ 5' and 3' non-coding regions (which had been PCR-amplified) upstream and downstream of the *ura4*$^+$ gene, which was cloned in a KS+ vector.

Protein analysis

Induction of mating in liquid medium and Western blotting was performed as described previously (Sharifmoghadam & Valdivieso, 2008). In order to detect the GFP-tagged Dni1 protein, cells were broken in 50mM Tris HCl, pH 7.5, 300 mM NaCl, 50 mM EDTA supplemented with protease inhibitors (1 mM PMSF; 1 μg/ml Aprotinin, Leupeptin and Pepstatin), and cell debris was eliminated by centrifuging at 1600 rpm for 5 minutes at 4ºC. Then, cell extracts were concentration-equalled (total protein was estimated using the Biorad protein assay kit; Bradford method), incubated at 0ºC in the presence of 1.6 M urea for 20 minutes and centrifuged at 13000 rpm for 30 minutes at 4ºC. Samples were denatured in Laemmli sample buffer (50 mM HCl-Tris, pH 6.8; 1% SDS; 143 mM β-mercaptoethanol; 10% glycerol) at 42ºC for 5 minutes. In order to identify unspecific bands and bands corresponding to cleaved GFP cell extracts form a strain that carried a soluble GFP under the control of the *map4*$^{+}$ promoter were analyzed in parallel. Anti-GFP and anti-Cdc2 were used at 1:1000 and 1:4000 dilutions, respectively.

Calcium accumulation analysis

Calcium uptake and accumulation was measured as previously described (Iida *et al.*, 1994, Paidhungat & Garrett, 1997). Basically, *cyr1Δ sxa2Δ* cells were incubated in EMM or in EMM-Ca for four hours at 30ºC. Then, 3.8 μl of $^{45}Ca^{++}$ (CES3, Amersham; 2.2 μCi/μl) was added, the cells were treated or not with factor P as described above, and incubated for different times at 32ºC. The reaction was stopped by mixing 0.2 ml samples from the cultures with 2 ml of cold STOP buffer (100 mM Tris-HCl, pH 6.8/ 20 mM $CaCl_2$). Samples were filtered and radioactivity was measured using a liquid scintillation counter. Non-radioactive cultures were performed in parallel and were used to estimate the cell number in each sample.

Microscopy

A Leica DM RXA microscope equipped with a Photometrics Sensys CCD camera using the Qfish 2.3 program was used to perform optic microscopy analyses. Calcofluor (BLANKOPHOR, Bayer) and DAPI (SIGMA) staining was performed as described (Arellano *et al.*, 2000). For FITC-conjugated concanavalin A (SIGMA) staining, cells were washed twice with 1ml of phosphate-buffered saline (PBS) and suspended in 50 µl PBS. FITC–concanavalin A (5 µl of a stock solution at 1mg/ml) was added and the cells were incubated at room temperature for 15 min in the dark. After incubation, the cells were washed three times with 500 µl of PBS, resuspended in 50 µl of the same buffer, and observed under a fluorescence microscope. Actin staining with rhodamine-phalloidin was performed as in (Marks & Hyams, 1985), and anti-tubulin immunofluorescence was performed by fixing the cells in methanol as described in (Hagan & Hyams, 1988) with an anti-Tat1 antibody. Cell lysis was assessed by resuspending the cells in 0.3% methylene blue and immediately observing them under the bright-field microscope. FM4-64 (BIOTIUM) staining was performed by adding this dye at a final concentration of 4 µM to the samples, incubating them for different times and washing the cells with cold EMM. Filipin (SIGMA) staining and treatment of cells was performed as described (Wachtler *et al.*, 2003). Methyl-β-cyclodextrins (SIGMA) were used at 200 mM. For transmission electron microscopy, cells were fixed with 2% EM grade glutaraldehyde (GA; Electron Microscopy Science) in 50 mM phosphate buffer, pH 7.2, 150 mM NaCl (PBS) for 2 h at 4°C, post-fixed with 1.2% potassium permanganate overnight at 4°C and embedded in Quetol 653, as described (Konomi *et al.*, 2003). Ultrathin sections were stained in 4% uranyl acetate and 0.4% lead citrate, and viewed under a TEM H-800 (Hitachi) operating at 125 keV.

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References

Aguilar, P.S., Engel, A. & Walter, P., (2007) The plasma membrane proteins Prm1 and Fig1 ascertain fidelity of membrane fusion during yeast mating. *Mol Biol Cell* **18**: 547-556.

Alvarez, F.J., Douglas, L.M., Rosebrock, A. & Konopka, J.B., (2008) The Sur7 protein regulates plasma membrane organization and prevents intracellular cell wall growth in *Candida albicans*. *Mol Biol Cell* **19**: 5214-5225.

Arcangioli, B. & Thon, G., (2004) Mating-Type Cassettes: Structure, Switchin and silencing. In: The molecular Biology of *Schizosaccharomyces pombe.* R. Egel (ed). Heidelberg: Springer, pp. 9-147.

Arellano, M., Cartagena-Lirola, H., Nasser Hajibagheri, M.A., Durán, A. & Valdivieso, M.H., (2000) Proper ascospore maturation requires the *chs1*$^{+}$ chitin synthase gene in *Schizosaccharomyces pombe*. *Mol Microbiol* **35**: 79-89.

Bagnat, M. & Simons, K., (2002) Cell surface polarization during yeast mating. *Proc Natl Acad Sci U S A* **99**: 14183-14188.

Bähler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., McKenzie, A., 3rd, Steever, A.B., *et al.*, (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* **14**: 943-951.

Brand, A., Shanks, S., Duncan, V.M., Yang, M., Mackenzie, K. & Gow, N.A., (2007) Hyphal orientation of *Candida albicans* is regulated by a calcium-dependent mechanism. *Curr Biol* **17**: 347-352.

Brand, A., Lee, K., Veses, V. & Gow, N.A., (2009) Calcium homeostasis is required for contact-dependent helical and sinusoidal tip growth in *Candida albicans* hyphae. *Mol Microbiol* **71**: 1155-1164.

Calleja, G., Bong, Y. & Johnson, B., (1977a) Fusion and erosion of cell walls during conjugation in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci.* **25**: 139-155.

Calleja, G.B. & Johnson, B.F., (1971) Flocculation in a fission yeast: an initial step in the conjugation process. *Can J Microbiol* **17**: 1175-1177.

Calleja, G.B., Yoo, B.Y. & Johnson, B.F., (1977b) Conjugation-induced lysis of *Schizosaccharomyces pombe*. *J. Bacteriol.* **130**: 512-515.

Calleja, G.B., (1987) Cell agregation. In: The Yeasts. A. H. Rose & J. S. Harrison (eds). London: Academic Press, pp. 165-238.

Carnero, E., Ribas, J.C., Garcia, B., Duran, A. & Sanchez, Y., (2000) *Schizosaccharomyces pombe* ehs1p is involved in maintaining cell wall integrity and in calcium uptake. *Mol Gen Genet* **264**: 173-183.

Cartagena-Lirola, H., Duran, A. & Valdivieso, M.H., (2006) The *Schizosaccharomyces pombe cfr1*$^{+}$ gene participates in mating through a new pathway that is independent of *fus1*$^{+}$. *Yeast* **23**: 375-388.

Davey, J., (1998) Fusion of a fission yeast. *Yeast* **14**: 1529-1566.

Drabikowski, W., Lagwinska, E. & Sarzala, M.G., (1973) Filipin as a fluorescent probe for the location of cholesterol in the membranes of fragmented sarcoplasmic reticulum. *Biochimica et biophysica acta* **291**: 61-70.

Egel, R., Willer, M., Kjaerulff, S., Davey, J. & Nielsen, O., (1994) Assessment of pheromone production and response in fission yeast by a halo test of induced sporulation. *Yeast* **10**: 1347-1354.

Erdman, S., Lin, L., Malczynski, M. & Snyder, M., (1998) Pheromone-regulated genes required for yeast mating differentiation. *J Cell Biol.* **140**: 461-483.

Gammie, A.E., Brizzio, V. & Rose, M.D., (1998) Distinct morphological phenotypes of cell fusion mutants. *Mol Biol Cell* **9**: 1395-1410.

Gonzalez-Mariscal, L., Betanzos, A., Nava, P. & Jaramillo, B.E., (2003) Tight junction proteins. *Progress in biophysics and molecular biology* **81**: 1-44.

Grossmann, G., Malinsky, J., Stahlschmidt, W., Loibl, M., Weig-Meckl, I., Frommer, W.B., *et al.*, (2008) Plasma membrane microdomains regulate turnover of transport proteins in yeast. *J Cell Biol*.

Hagan, I.M. & Hyams, J.S., (1988) The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast *Schizosaccharomyces pombe*. *J Cell Sci* **89 (Pt 3)**: 343-357.

Heiman, M.G. & Walter, P., (2000) Prm1p, a pheromone-regulated multispanning membrane protein, facilitates plasma membrane fusion during yeast mating. *J Cell Biol* **151**: 719-730.

Heiman, M.G., Engel, A. & Walter, P., (2007) The Golgi-resident protease Kex2 acts in conjunction with Prm1 to facilitate cell fusion during yeast mating. *J Cell Biol* **176**: 209-222.

Hemler, M.E., (2005) Tetraspanin functions and associated microdomains. *Nature reviews* **6**: 801-811.

Iida, H., Yagawa, Y. & Anraku, Y., (1990) Essential role for induced Ca2+ influx followed by [Ca2+]i rise in maintaining viability of yeast cells late in the mating pheromone response pathway. A study of [Ca2+]i in single *Saccharomyces cerevisiae* cells with imaging of fura-2. *J Biol Chem* **265**: 13391-13399.

Iida, H., Nakamura, H., Ono, T., Okumura, M.S. & Anraku, Y., (1994) *MID1*, a novel *Saccharomyces cerevisiae* gene encoding a plasma membrane protein, is required for Ca^{2+} influx and mating. *Mol Cell Biol* **14**: 8259-8271.

Jin, H., Carlile, C., Nolan, S. & Grote, E., (2004) Prm1 prevents contact-dependent lysis of yeast mating pairs. *Eukaryot Cell* **3**: 1664-1673.

Jin, H., McCaffery, J.M. & Grote, E., (2008) Ergosterol promotes pheromone signaling and plasma membrane fusion in mating yeast. *J Cell Biol* **180**: 813-826.

Kaji, K. & Kudo, A., (2004) The mechanism of sperm-oocyte fusion in mammals. *Reproduction* **127**: 423-429.

Konomi, M., Fujimoto, K., Toda, T. & Osumi, M., (2003) Characterization and behaviour of alpha-glucan synthase in *Schizosaccharomyces pombe* as revealed by electron microscopy. *Yeast* **20**: 427-438.

Kurahashi, H., Imai, Y. & Yamamoto, M., (2002) Tropomyosin is required for the cell fusion process during conjugation in fission yeast. *Genes Cells.* **7**: 375-384.

Marks, J. & Hyams, J.S., (1985) localisation of F-actin through the cell division cycle of *Schizosaccharomyces pombe*. *Eur J Cell Biol* **39**: 27-32.

Marsh, L. & Rose, M.D., (1997) The pathway of cell and nuclear fusion during mating in *S. cerevisiae*. In: The molecular and cellular biology of the yeast *Saccharomyces*. J. R. Pringle, Broach, J. R., Jones, E. W. (ed). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, pp. 1037-1106.

Mata, J., Lyne, R., Burns, G. & Bähler, J., (2002) The transcriptional program of meiosis and sporulation in fission yeast. *Nature Genet.* **32**: 143-147.

Mata, J. & Bahler, J., (2006) Global roles of Ste11p, cell type, and pheromone in the control of gene expression during early sexual differentiation in fission yeast. *Proc Natl Acad Sci U S A* **103**: 15517-15522.

Moreno, S., Klar, A. & Nurse, P., (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol* **194**: 795-823.

Muller, E.M., Locke, E.G. & Cunningham, K.W., (2001) Differential regulation of two Ca(2+) influx systems by pheromone signaling in *Saccharomyces cerevisiae*. *Genetics.* **159**: 1527-1538.

Muller, E.M., Mackin, N.A., Erdman, S.E. & Cunningham, K.W., (2003) Fig1p facilitates Ca2+ influx and cell fusion during mating of *Saccharomyces cerevisiae*. *J Biol Chem.* **278**: 38461-38469.

Nakamura, T., Asakawa, H., Nakase, Y., Kashiwazaki, J., Hiraoka, Y. & Shimoda, C., (2008) Live observation of forespore membrane formation in fission yeast. *Mol Biol Cell* **19**: 3544-3553.

Nielsen, O., (2004) Mating-type control and differentiation. In: The molecular biology of *Schizosaccharomyces pombe*. R. Egel (ed). Heidelberg: Springer, pp. 281-296.

Paidhungat, M. & Garrett, S., (1997) A homolog of mammalian, voltage-gated calcium channels mediates yeast pheromone-stimulated Ca2+ uptake and exacerbates the cdc1(Ts) growth defect. *Mol Cell Biol* **17**: 6339-6347.

Pelham, R.J., Jr. & Chang, F., (2001) Role of actin polymerization and actin cables in actin-patch movement in *Schizosaccharomyces pombe*. *Nat Cell Biol* **3**: 235-244.

Petersen, J., Weilguny, D., Egel, R. & Nielsen, O., (1995) Characterization of *fus1* of *Schizosaccharomyces pombe*: a developmentally controlled function needed for conjugation. *Mol Cell Biol* **15**: 3697-3707.

Petersen, J., Heitz, M.J. & Hagan, I.M., (1998a) Conjugation in *S. pombe*: identification of a microtubule-organising centre, a requirement for microtubules and a role for Mad2. *Curr. Biol.* **8**: 963-966.

Petersen, J., Nielsen, O., Egel, R. & Hagan, I.M., (1998b) F-actin distribution and function during sexual differentiation in *Schizosaccharomyces pombe*. *J Cell Sci* **111**: 867-876.

Petersen, J., Nielsen, O., Egel, R. & Hagan, I.M., (1998c) FH3, a domain found in formins, targets the fission yeast formin Fus1 to the projection tip during conjugation. *J Cell Biol* **141**: 1217-1228.

Proszynski, T.J., Klemm, R., Bagnat, M., Gaus, K. & Simons, K., (2006) Plasma membrane polarization during mating in yeast cells. *J Cell Biol* **173**: 861-866.

Sharifmoghadam, M.R., Bustos-Sanmamed, P. & Valdivieso, M.H., (2006) The fission yeast Map4 protein is a novel adhesin required for mating. *FEBS Lett* **580**: 4457-4462.

Sharifmoghadam, M.R. & Valdivieso, M.H., (2008) The *Schizosaccharomyces pombe* Map4 adhesin is a glycoprotein that can be extracted from the cell wall with

alkali but not with beta-glucanases and requires the C-terminal DIPSY domain for function. *Mol Microbiol* **69**: 1476-1490.

Shimoda, C. & Nakamura, T., (2004) Control of late meiosis and ascospore formation. In: The molecular biology of *Schizosaccharomyces pombe*. R. Egel (ed). Heidelberg: Springer, pp. 311-328.

Stein, K.K., Primakoff, P. & Myles, D., (2004) Sperm-egg fusion: events at the plasma membrane. *J Cell Sci* **117**: 6269-6274.

Stern, B. & Nurse, P., (1997) Fission yeast pheromone blocks S-phase by inhibiting the G1 cyclinB-p34^{cdc2} kinase. *EMBO J.* **16**: 534.

Stipp, C.S., Kolesnikova, T.V. & Hemler, M.E., (2003) Functional domains in tetraspanin proteins. *Trends Biochem Sci* **28**: 106-112.

Streiblová, E. & Wolf, A., (1975) Role of cell wall topography in conjugation in *Schizosaccharomyces pombe*. *Can. J. Microbiol.* **21**: 1399-1405.

Sutovsky, P., (2009) Sperm-egg adhesion and fusion in mammals. *Expert reviews in molecular medicine* **11**: e11.

Takeo, K., (1985) A correlation between mode of growth and regional ultrastructure of the plasma membrane of *Schizosaccharomyces pombe* as revealed by freeze-fracturing before and after filipin treatment. *Journal of general microbiology* **131**: 309-316.

Tarrant, J.M., Robb, L., van Spriel, A.B. & Wright, M.D., (2003) Tetraspanins: molecular organisers of the leukocyte surface. *Trends in immunology* **24**: 610-617.

Tasaka, Y., Nakagawa, Y., Sato, C., Mino, M., Uozumi, N., Murata, N., *et al.*, (2000) *yam8*(+), a *Schizosaccharomyces pombe* gene, is a potential homologue of the *Saccharomyces cerevisiae MID1* gene encoding a stretch-activated Ca(2+)-

permeable channel. *Biochemical and biophysical research communications* **269**: 265-269.

Wachtler, V., Rajagopalan, S. & Balasubramanian, M.K., (2003) Sterol-rich plasma membrane domains in the fission yeast *Schizosaccharomyces pombe*. *J Cell Sci* **116**: 867-874.

Walther, T.C., Brickner, J.H., Aguilar, P.S., Bernales, S., Pantoja, C. & Walter, P., (2006) Eisosomes mark static sites of endocytosis. *Nature* **439**: 998-1003.

Wassarman, P., (1999) Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis and fusion. *Cell* **96**: 175-183.

Yamamoto, M., Imai, Y. & Watanabe, Y., (1997) Mating and sporulation in *Schizosaccharomyces pombe*. In: The molecular and cellular biology of the yeast *Saccharomyces*. J. R. Pringle, J. R. Broach & E. W. Jones (eds). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, pp. 1037-1106.

Yamamoto, M., (2004) Initiation of meiosis. In: The molecular Biology of *Schizosaccharomyces pombe*. R. Egel (ed). Heidelberg: Springer, pp. 297-310.

Ydenberg, C.A. & Rose, M.D., (2008) Yeast mating: a model system for studying cell and nuclear fusion. *Methods in molecular biology (Clifton, N.J* **475**: 3-20.

Yunta, M. & Lazo, P.A., (2003) Tetraspanin proteins as organisers of membrane microdomains and signalling complexes. *Cell Signal* **15**: 559-564.

Zhang, N.N., Dudgeon, D.D., Paliwal, S., Levchenko, A., Grote, E. & Cunningham, K.W., (2006) Multiple signaling pathways regulate yeast cell death during the response to mating pheromones. *Mol Biol Cell* **17**: 3409-3422.

Figure legends

Figure 1-Multiple sequence alignment of the region surrounding the Cys-containing motif of the indicated proteins. The aminoacids coincident with the Fig1-family consensus, GΦΦGXC(8-20 aa)C, have been shadowed or underlined. Complete sequence alignment between Fig1p, Dni1p and Dni2p is shown in Supplementary figure S1.

Figure 2- Dni1p is expressed during mating. (A) Levels of GFP-fused Dni1p (left-hand lanes) in cells that had been incubated in minimal medium without nitrogen for the indicated time (hours). The arrow points to the band corresponding to Dni1p. The level of the GFP alone, expressed under the control of the *map4*$^{+}$ promoter, after 0 and 6 hours in minimal medium without nitrogen is shown (right-hand lanes). The level of Cdc2p was used as a loading control. The molecular weight is indicated in kilodalton (KDa). The percentage of zygotes (Z, %) and asci (A, %) in the cultures are indicated. (B-E) Localization of Dni1p. (B) Dni1p localization to the tip of the shmoos. (C) Dni1p localization to the cell-cell contact area of prezygotes in which the cell wall separating the mating cells has not yet been digested; panel i shows a prezygote exhibiting the typical localization of this protein; panel ii shows a prezygote in which Dni1p is localized throughout the cell body, and panel iii shows a prezygote in which Dni1p (indicated by the arrow) spreads along the plasma membrane at the area of cell-cell contact of the mating cells. (D) Localization of Dni1p at the mating bridge of zygotes. (E) Localization of Dni1p in *asci*. The drawings depict the morphology of the cell type photographed. Bar, 10 μM.

Figure 3- Dni1 localization to the tip of the shmoos depends on Fus1p, actin, and lipid rafts. (A) Dni1-GFP localization in zygotes and shmoos (inset in the h^{90} *fus1Δ* panel) of the indicated strains incubated in EMM at 32ºC for two days. (B) Fus1-GFP localization in h^{90} and h^{90} *dni1Δ* cells. (C) *cyr1Δ sxa2Δ* cells carrying the GFP-fused Dni1 protein were treated with the solvent DMSO or with 100 µM latrunculin A for 10 minutes and photographed. (D) *cyr1Δ sxa2Δ* cells carrying either GFP-Bgs4 (left panels) or Dni1-GFP (right panels) were stained with 5 µg/µl filipin for 1 minute (upper panels) or treated with this compound for one hour (lower panels). For each experiment the fluorescence corresponding to sterols (filipin) or to proteins (GFP-Bgs4 and Dni1-GFP) is shown.

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Figure 5- Electron microscopy of h^{90} (A) and h^{90} *dni1Δ* (B) zygotes. Cell wall (cw), plasma membrane (mb), and cytoplasm (cyt) are indicated by arrows in the regions of interest.

Figure 6- Cell fusion is sensitive to temperature in *dniΔ* and *dni2Δ* mutants. (A) Sporulation efficiency (*asci* with respect to zygotes plus *asci*) of the indicated h^{90} strains incubated at the indicated temperatures for 4 days. (B) Sporulation efficiency (*asci* with respect to total cell number) of the indicated diploid strains incubated at the indicated temperatures for 4 days. The experiments were performed three times. Standard deviations are shown. (C) Sporulation efficiency of the indicated h^{90} strains incubated at the indicated temperatures for 4 days. (D) Overlapping images of bright-field and fluorescence images (BF+GFP) of h^{90} *dni2Δ* and h^{90} *dni1Δ dni2Δ* zygotes expressing a soluble GFP under the control of the h^{+}-specific agglutinin *map4*$^{+}$

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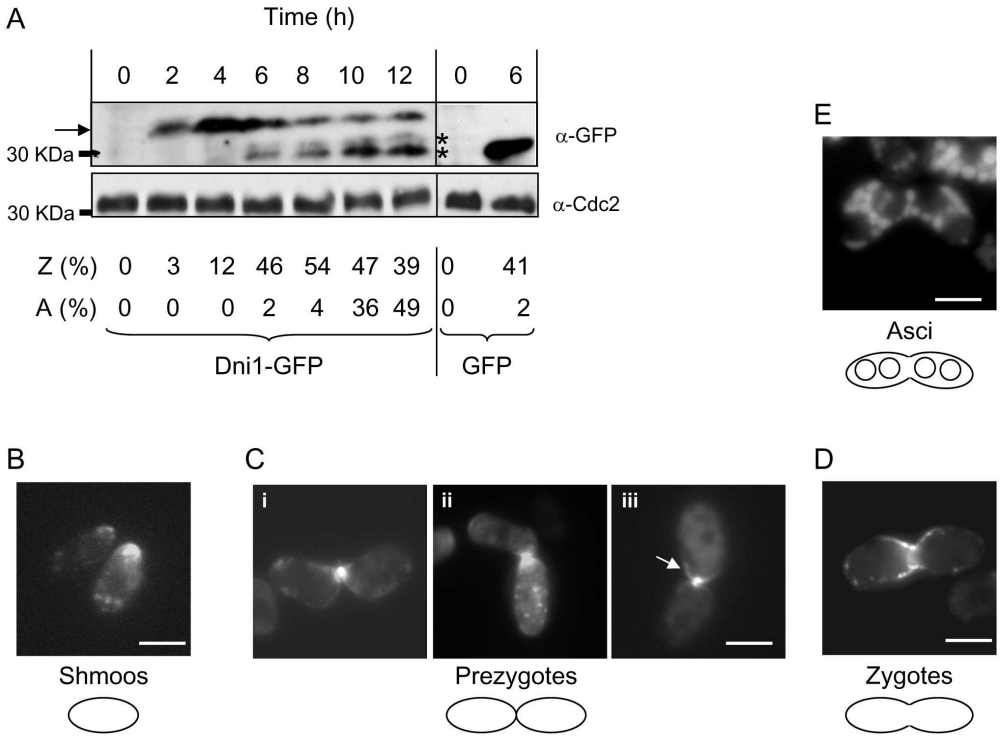

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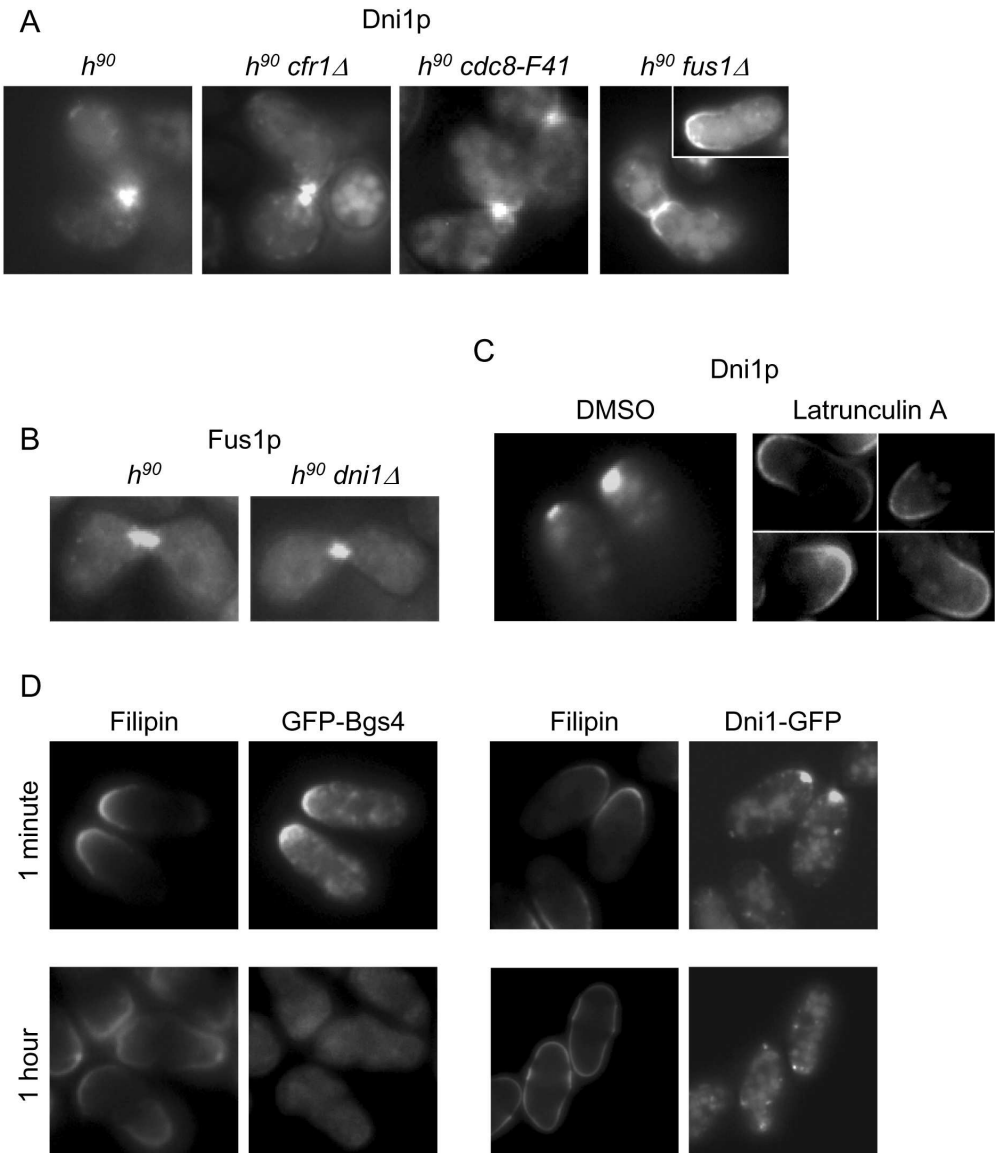

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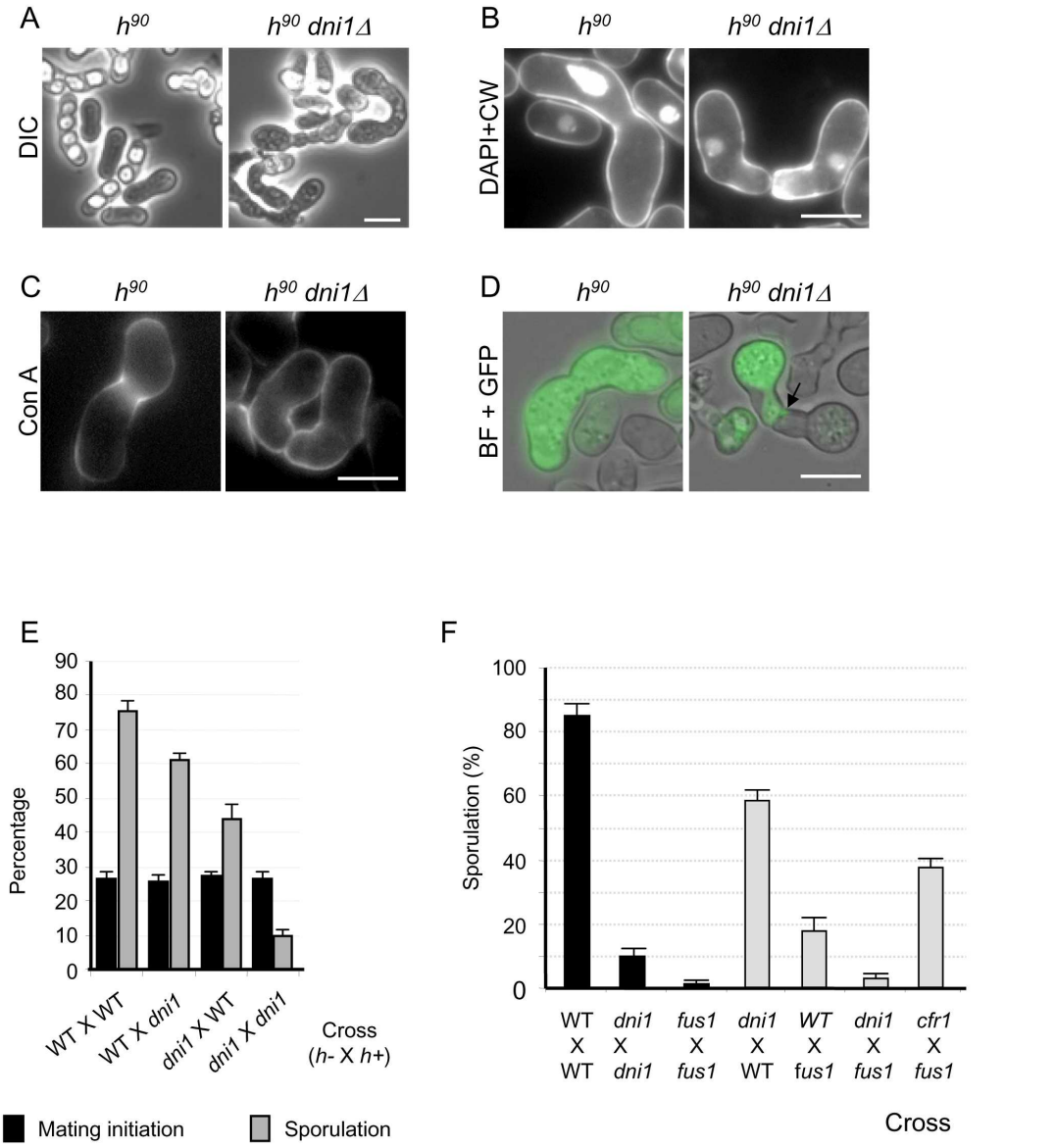

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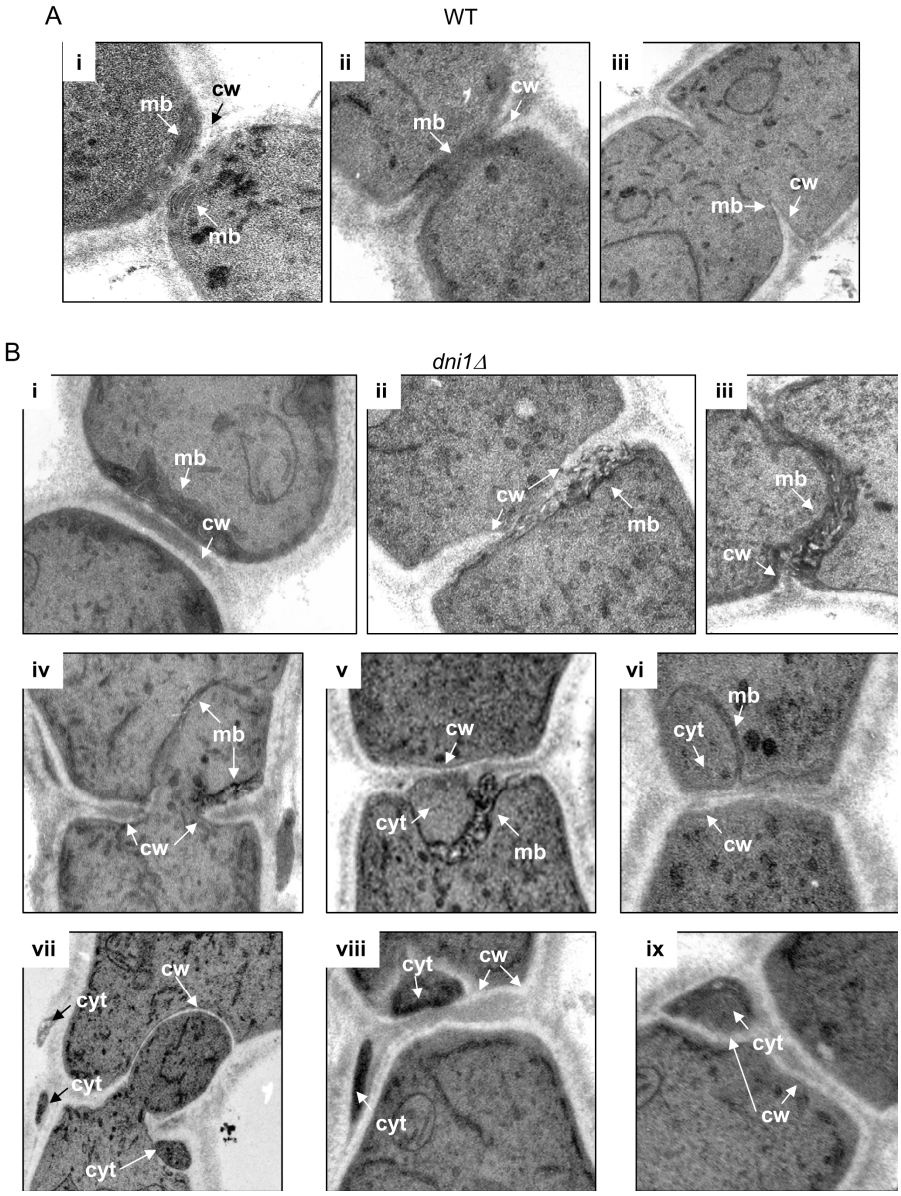

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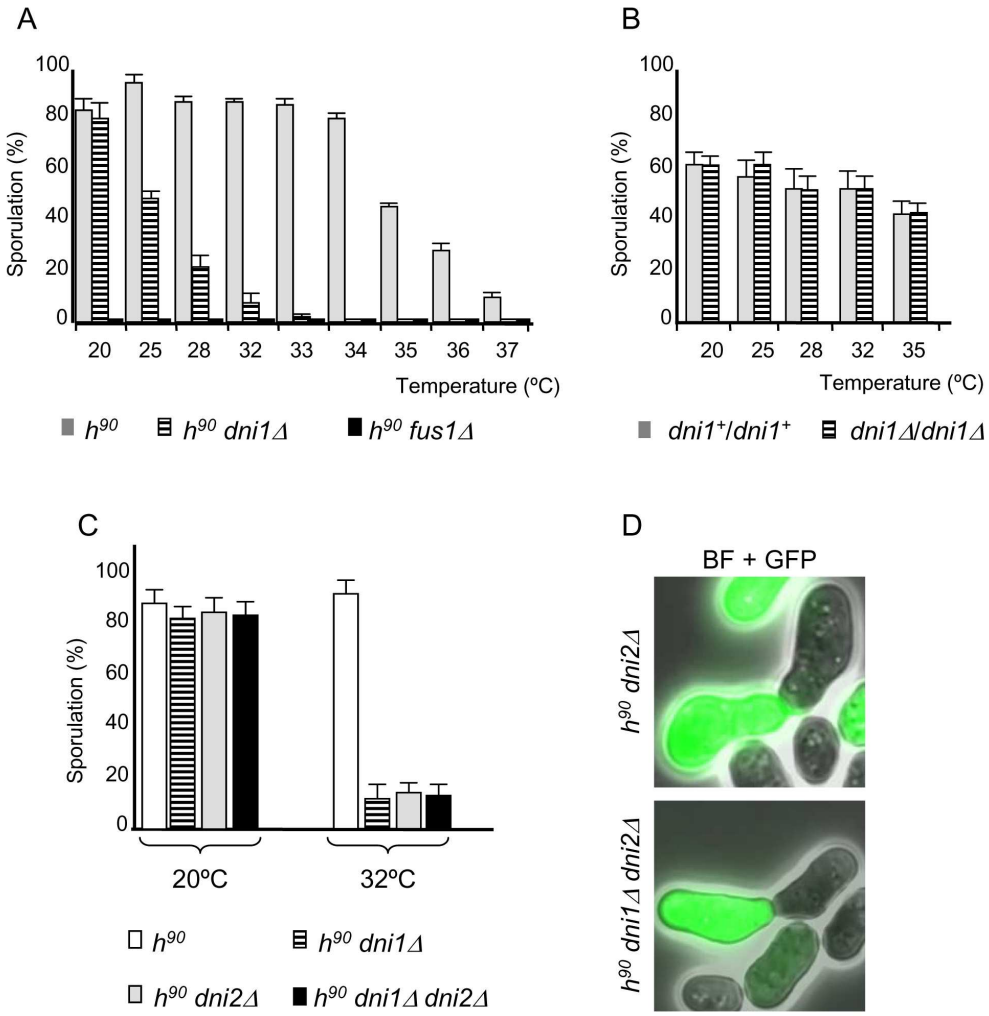

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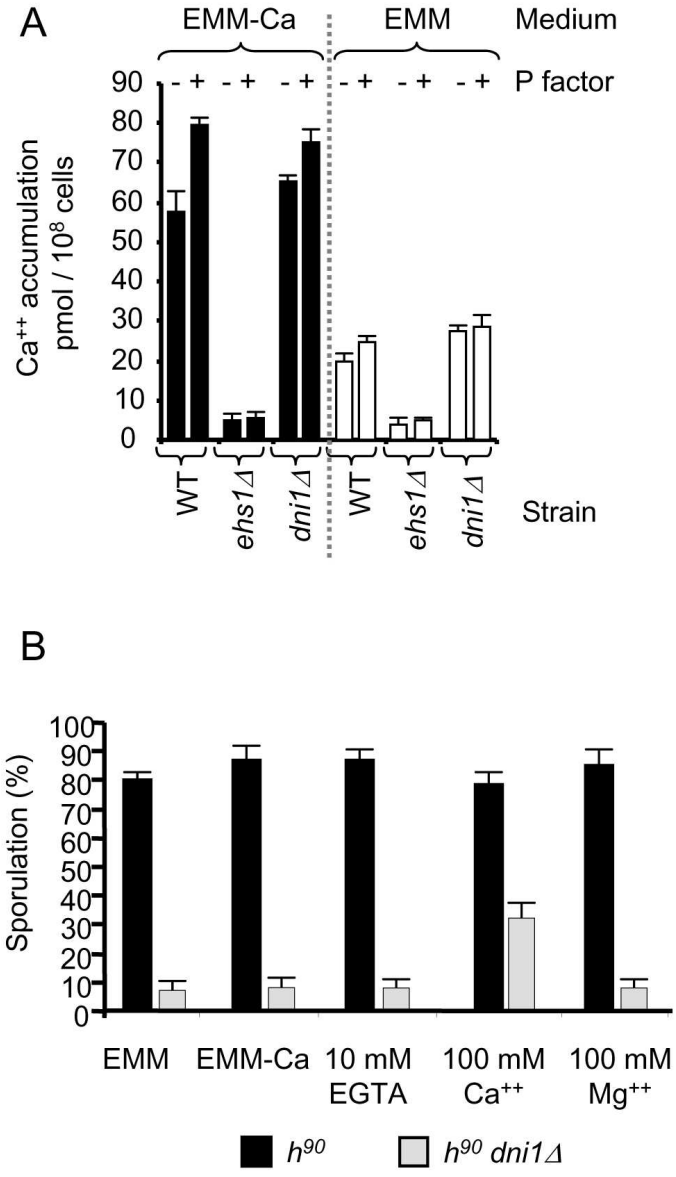

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