A CELL FUSION PROTEIN IN YEAST INTERACTS WITH PROTEINS REQUIRED FOR MATING AND POLARIZED GROWTH

by

Steven John Ritchie
B.Sc., Oxford Brookes University, 1992

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE in the Department of Biological Sciences

© Steven John Ritchie 1997

SIMON FRASER UNIVERSITY
September 1997

All rights reserved. This work may not be reproduced in whole or in part, by photocopy or other means, without permission of the author.
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-24231-5
APPROVAL

Name: Steven John Ritchie

Degree: MASTER OF SCIENCE

Title of Thesis:
A Cell Fusion Protein in Yeast Interacts with Proteins Required for Mating and Polarized Growth.

Examining Committee:
Chair: Dr. F. Breden, Associate Professor

Dr. C. Boone, Assistant Professor, Senior Supervisor
Department of Biological Sciences, S.F.U.

Dr. D. Baillie, Professor
Department of Biological Sciences, S.F.U.

Dr. J. Price, Assistant Professor
Department of Biological Sciences, S.F.U.

Dr. M. E. Glass, Associate Professor
Department of Biotechnology/Botany, U.B.C.
Public Examiner

Date Approved: Sept. 17, 1997
Saccharomyces cerevisiae provides an elegant model for the study of cell conjugation and fusion. Fus1p is required for efficient cell fusion during the mating process, and localizes to the cell surface. It is strongly induced in response to pheromone, possesses a single transmembrane domain, is O-glycosylated, and contains one SH3 (src-homology three) domain at the C-terminus of its cytoplasmic portion. In this study I adopted the two-hybrid system to test Fus1p for interaction with various proteins involved in cell fusion and signalling. Fus1p interacted with Rvs167p, a cytoskeletal associated protein, Fus2p, a second pheromone inducible and fusion related protein, Fus3p, Kss1p, Ste11p, Ste5p, and Ste7p, components of the MAPK pheromone response pathway, and Chs5p, a protein that is potentially involved in vesicular transport. I also screened two-hybrid libraries with the cytoplasmic portion of Fus1p, including the SH3 domain. I isolated Bud2p, the GTPase activating protein for Rsr1p, Bnr1p, a formin homolog, and Ynl075wp, Ynl054wp, and Ylr117cp, three previously unidentified ORF encoded proteins. Further two-hybrid experiments demonstrated that Bnr1p interacts with Axl1p, a protein responsible for the haploid specific axial budding pattern. I term the complex of Fus1p and aforementioned proteins a "fusion complex". I propose that the "fusion complex" links the pheromone response pathway to proteins that participate in both reorganization of the actin cytoskeleton and endocytotic vesicular recycling.
DEDICATION

This thesis is dedicated to my parents and especially my girlfriend, Sanya.
ACKNOWLEDGEMENTS

I would like to express my thanks to my senior supervisor Dr. Charles Boone for accepting me into his laboratory, teaching me the fundamentals of yeast genetics and providing a wealth of ideas and encouragement throughout my work. I would also like to thank my supervisory committee members Dr. David Baillie and Dr. James Price for their ideas and support on my project. And thanks to all the members of the Boone Lab, past and present, especially fellow graduate students Neil Adames and Pradeep Nair, Lana Pratt for her work on the two-hybrid screens, Kelly Blundell, Marie Evangelista, Bryce Nelson, Isabelle Pot, Grant Poje and the rest for their help and humour.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPROVAL PAGE</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae as a model organism</td>
<td>1</td>
</tr>
<tr>
<td>The mating reaction</td>
<td>2</td>
</tr>
<tr>
<td>The MAPK cascade</td>
<td>3</td>
</tr>
<tr>
<td>The S. cerevisiae pheromone response MAPK cascade</td>
<td>3</td>
</tr>
<tr>
<td>Pheromone inducible genes (PREs)</td>
<td>6</td>
</tr>
<tr>
<td>Polarity establishment</td>
<td>8</td>
</tr>
<tr>
<td>The budding program</td>
<td>8</td>
</tr>
<tr>
<td>The mating program</td>
<td>9</td>
</tr>
<tr>
<td>Bni1p</td>
<td>11</td>
</tr>
<tr>
<td>Conjugation</td>
<td>12</td>
</tr>
<tr>
<td>Genes involved in nuclear fusion</td>
<td>12</td>
</tr>
<tr>
<td>Genes involved in cell fusion</td>
<td>13</td>
</tr>
<tr>
<td>SH3 domains</td>
<td>18</td>
</tr>
<tr>
<td>The RVS gene family</td>
<td>19</td>
</tr>
<tr>
<td>Chitin synthase 5</td>
<td>22</td>
</tr>
<tr>
<td>Two-Hybrid system</td>
<td>25</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>28</td>
</tr>
<tr>
<td>Strains and media</td>
<td>28</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Plasmid construction</td>
<td>30</td>
</tr>
<tr>
<td>Yeast methods and transformations</td>
<td>36</td>
</tr>
<tr>
<td>Molecular biological techniques</td>
<td>36</td>
</tr>
<tr>
<td>PCR check of $bnr1\Delta::URA3$ gene disruption strains</td>
<td>37</td>
</tr>
<tr>
<td>Multicopy suppression screen of $fus1\Delta sst2\Delta$</td>
<td>37</td>
</tr>
<tr>
<td>Yeast two-hybrid method</td>
<td>37</td>
</tr>
<tr>
<td>Pheromone response assay</td>
<td>38</td>
</tr>
<tr>
<td>G1 arrest plate assay</td>
<td>39</td>
</tr>
<tr>
<td><strong>RESULTS</strong></td>
<td>40</td>
</tr>
<tr>
<td>Multicopy suppressor screen in a $fus1\Delta sst2\Delta$ background</td>
<td>40</td>
</tr>
<tr>
<td>Two-Hybrid experiments with Fuslp and proteins</td>
<td>43</td>
</tr>
<tr>
<td>involved in reorganization of the actin cytoskeleton</td>
<td></td>
</tr>
<tr>
<td>Genetic experiments with $RV5167$</td>
<td>46</td>
</tr>
<tr>
<td>Two-Hybrid experiments with Fuslp and the MAPK</td>
<td>47</td>
</tr>
<tr>
<td>cascade pathway components</td>
<td></td>
</tr>
<tr>
<td>Two-Hybrid Library search with Fuslp as bait</td>
<td>49</td>
</tr>
<tr>
<td>Gene disruptions of $BNR1, YNL054w, YNL075w$ and $YLR117c$</td>
<td>52</td>
</tr>
<tr>
<td>Gene disruption of $BNR1$</td>
<td>56</td>
</tr>
<tr>
<td>Gene disruptions of $YNL054w, YNL075w, and YLR117c$</td>
<td>59</td>
</tr>
<tr>
<td>Two-Hybrid experiments with Bnr1p</td>
<td>59</td>
</tr>
<tr>
<td>Two-Hybrid experiments with Chs5p</td>
<td>60</td>
</tr>
<tr>
<td><strong>DISCUSSION</strong></td>
<td>62</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>77</td>
</tr>
</tbody>
</table>
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table I</td>
<td>Strains</td>
<td>28</td>
</tr>
<tr>
<td>Table II</td>
<td>PCR primers and oligonucleotides</td>
<td>30</td>
</tr>
<tr>
<td>Table III</td>
<td>Plasmids</td>
<td>32</td>
</tr>
<tr>
<td>Table IV</td>
<td>Multicopy suppressors of the Y106 fus1Δst2Δ</td>
<td>42</td>
</tr>
<tr>
<td>Table V</td>
<td>Interactions of Fus1p (97-512aa), p1336, with Rvs167p (p627), Fus1p (p628), Srv2p (p625), Rvs161p (p974), profilin (p624), actin (p626) and pACT2 (p892).</td>
<td>42</td>
</tr>
<tr>
<td>Table VI</td>
<td>Interactions of Fus1p (97-512aa), p1336, with the MAPK pheromone response pathway components and other cytoskeletal proteins in wild-type (Y704), ste7Δ (Y1097), and ste11Δ (Y1098) backgrounds.</td>
<td>48</td>
</tr>
<tr>
<td>Table VII</td>
<td>Interactions of Fus1p (97-512aa), p1336, with Bnr1p (297-789aa), Bud2p (849-1105aa), Ynl054wp (665-916aa), Ylr117cp (274-573aa) and Ynl075wp (38-291aa) isolated from a 2-hybrid library screen with Fus1p (97-512aa) as 'bait'.</td>
<td>53</td>
</tr>
<tr>
<td>Table VIII</td>
<td>Tetrad data from Y688 (bni1Δ:LEU2/BNI1+ a/α diploid) transformed with EcoRI digested p1527 (b:n1Δ::URA3) gene disruption plasmid, sporulated and tetrads dissected.</td>
<td>57</td>
</tr>
<tr>
<td>Table IX</td>
<td>Interactions of Chs5p (p1608) with the MAPK pheromone response pathway components and other cytoskeletal proteins.</td>
<td>61</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

Figure 1: The *S. cerevisiae* MAPK pheromone response cascade ............... 4

Figure 2: The structure of Fus1p ................................................................ 14

Figure 3: A *FUS1*-Green Fluorescent Protein fusion expressed ............... 16

in a wild-typeMATa cell responding to an exogenous 
source of α-factor pheromone.

Figure 4: Qualitative mating assay of Y106 fus1Δsst2Δ ......................... 45

transformed with YEp24 vector (p5), YEp24::*FUS1* (p264),
YEp24::*FUS2* (p304), YEp24::*MFA1* (p471) and wild-type
(W3031A), mated to a wild-type strain (Y63).

Figure 5: Qualitative mating assay of *fus1Δ sst2Δ* (Y106) ................. 45

wild-type (W3031A), *rvs167Δ rvs161Δ* (Y1096),
*rvs167Δ fus1Δ* (Y1117) and *rvs161Δ fus1Δ* (Y736), mated
to an enfeebled strain (Y66).

Figure 6: A schematic diagram showing the genes isolated ................. 51

from a two-hybrid library screen with Fus1p (97-512aa).

Figure 7: Hydrophobicity plot of the predicted protein ....................... 54

encoded by *YNL054w*.

Figure 8: An alignment of the proteins encoded by ............................ 54

*S. cerevisiae* *YNL075w* and *C. elegans* ZK795c.

Figure 9: An alignment of the proteins encoded by *S. cerevisiae* ......... 55

*YLR117c* and *D. melanogaster* crooked neck.

Figure 10: PCR check of *bnr1Δ* gene disruption strains ...................... 58

Figure 11: Qualitative mating assay of wild-type (W3031B) ................. 58

*bnr1Δ* (Y654), *bnr1Δ* (Y1007) and *bnr1Δbnr1Δ* (Y941)
mated to the wild type mating tester strain (Y62).
Figure 12: A model to show the putative "fusion-complex" formed between components of the MAPK pheromone response cascade, Fus1p, Fus2p; Rvs161p, Rvs167p and Chs5p, based upon two-hybrid analysis of protein-protein interactions.
INTRODUCTION

Saccharomyces cerevisiae as a model organism

The budding yeast Saccharomyces cerevisiae is one of the most well utilized and characterized model organisms in the study of a variety of cellular and molecular processes. The yeast is unicellular, grows rapidly on liquid and solid substrates, and has very well characterized genetics.

S. cerevisiae exists in both a stable haploid and diploid phase of its life cycle. Haploid cells exist as one of two mating types, designated MATa or MATα, determined by the mating-type cassette present at the MAT locus. The diploid phase, under conditions of nutrient and nitrogen starvation, enters meiosis to form an ascus containing four haploid spores. The ascus permits the recovery of all four meiotic products, and therefore has allowed for detailed genetic analysis and mapping to be performed. S. cerevisiae is also a powerful tool for molecular experiments. The ease with which homologous recombination occurs in yeast, allows for the efficient creation of gene disruptions and deletions. S. cerevisiae possesses sixteen chromosomes, as a haploid, for which the complete DNA sequence has been recently determined (Cherry, J. M. et al., 1995). Approximately six thousand genes are coded for by this sequence, the majority of which remain unstudied to date. The next project, in terms of genomic analysis, is to make disruptions of all the coding ORFs and study the phenotypes of the mutants in order to create a database of information covering the entire organism. This project, "EuroFan", is already underway in many yeast laboratories around the world.
The mating reaction

The two haploid mating types of \textit{S. cerevisiae}, \textit{MATa} and \textit{MATa}, secrete mating pheromone into their environment, \( a \) and \( \alpha \) factor respectively. Each mating type possesses a specific seven-pass transmembrane receptor that binds the opposing mating type pheromone, encoded by \textit{STE2} (\( a \)-factor) and \textit{STE3} (\( \alpha \)-factor). In response to mating pheromone, \textit{S. cerevisiae} arrest their cell-cycle in G1 and become polarized, forming an extended projection, or shmoo, towards the pheromone source (Sprague, G. F. and J. Thorner, 1992). Once the two mating types come into contact with one another at the tips of their respective mating projections they form what is known as the pre-zygote, whilst the cell walls and plasma membranes remain intact (Trueheart, J. \textit{et al.}, 1987). Subsequently, the cell walls and plasma membranes that formerly separated the cells are actively broken down and restructured. This allows for continuity of the cytoplasm between the two cells, and cell fusion to take place. Following cell fusion, formation of the zygote is completed when the two haploid nuclei fuse to form one single diploid nucleus. If placed under conditions of nutrient deprivation, the diploid zygote can subsequently enter meiosis and sporulation. Under rich nutrient conditions, the diploid cell will undergo consecutive rounds of mitotic division, ie. budding.

In this thesis I will focus on the mating reaction in \textit{S. cerevisiae}, and in particular the fusion event that takes place during the transition from pre-zygote to zygote.
The MAPK cascade

A mitogen-activated protein kinase (MAPK) cascade, the Pheromone Response Cascade, is responsible for the conversion of the extracellular pheromone signal into an intracellular signal, resulting in the transcription of mating specific genes and G1 arrest. MAPK cascades are composed of a family of eukaryotic protein kinases which control a variety of growth, differentiation and stress response pathways in eukaryotes, activated via a variety of extracellular signals binding to a variety of cell-surface bound receptors (including seven-pass transmembrane, cytokine, tyrosine kinase and two-component receptors). MAP kinases are activated via sequential phosphorylations of both tyrosine and threonine residues. The various cascades possess different upstream signals but all possess a structurally similar set of MAP kinase kinase kinase (MEKK), MAP kinase kinase (MEK) and a MAP kinase. Several MAPK cascades have been discovered, regulating a number of separate growth and differentiation pathways. The invasive (or pseudohyphal) response, high osmolarity glycerol response, maintenance of cell wall integrity, sporulation and mating response are all controlled by structurally similar, but functionally distinct MAPK cascades (Treisman, R., 1996; Waskiewicz, A. J. and J. A. Cooper., 1995).

The S. cerevisiae pheromone response MAPK cascade

The S. cerevisiae pheromone response MAPK cascade is composed of, at least, four protein kinases (Ste11p, Ste7p, Fus3p and Kss1p), with a scaffold-like protein Ste5p linking them together as a unit (see Figure 1). The pheromone receptor, encoded by STE2 in MATa and STE3 in MATα haploids, transmits a signal via association with a heterotrimeric G-protein, composed
Figure 1. The *S. cerevisiae* MAPK pheromone response cascade including a model for the role of Ste20p, Bem1p, Bni1p, Bud6p and Profilin in the rearrangement of the actin cytoskeleton (shaded)
of Gβ, Gα and Gγ sub-units. Activated receptors cause the Gα subunit to release GDP to bind GTP. This leads to dissociation of the Gβ·Gγ dimer which transmits the signal, via an as yet unknown mechanism, to the protein kinase Ste20p, a homolog of human p65PAK-type kinase (Manser, E. et al., 1994).

Ste20p, through an as yet unknown mechanism, activates the MEKK (MAPK kinase kinase) Ste11p, which in turn phosphorylates and activates the MEK Ste7p, which itself phosphorylates and activates the two MAP kinases Fus3p and Kss1p. Fus3p and Kss1p appear to be functionally redundant, they both transmit the signal by inducing cellular responses including: phosphorylation and activation of the transcription factor Ste12p, which activates transcription of many mating response genes, including FUS1 and FUS2; cell cycle arrest in G1 via inhibition of Cdc28p kinase by the cyclin-dependent kinase inhibitor Far1p; and promotes polarized morphogenesis.

The two homologous MAPKs Fus3p and Kss1p have overlapping functions in terms of signal transduction, a fus3Δ or kss1Δ can arrest in G1 and undergo the same morphological changes associated with pheromone response. However, FUS3 has a specific requirement in the cell fusion event, whereas KSS1 is only required in the signalling event. FUS3 has been described to play three roles in the cell conjugation process, G1 arrest, projection formation, and cell fusion (Elion, E. A. et al., 1990, 1991, 1993).

G1 arrest prior to START occurs by the pheromone induced expression of the Cdk (cyclin dependent kinase inhibitor) Far1p "factor arrest". G1 arrest in both mating partners ensures that they mate when they are in the same stage of the cell cycle, prior to DNA replication, i.e. as haploids. Far1p is activated by becoming phosphorylated on a number of residues (Chang, F. and I. Herskowitz, 1992). Fus3p kinase and the Cdc28p-Cln complexes are believed to carry out some of the phosphorylations that regulate Far1p

Phosphorylated Far1p induces G1 arrest by inhibiting the Cln1-Cdc28p and Cln2-Cdc28p kinase complexes (Peter, M. and Herskowitz, I., 1994). Studies expressing an allèle of STE3 that is resistant to α-factor (STE3^DAF) demonstrated that short term activation of Fus3p MAPK is sufficient for the early transcriptional response to pheromone, but sustained activation is required for cell cycle arrest (Couve, A. and Hirsch, J., 1996).

Restoration of the pathway to its inactivated state requires the Gβ•Gγ and Gα subunits to recombine. The Gα subunit possesses an intrinsic GTPase activity which catalyzes the conversion of bound GTP to GDP, the SST2 gene product, Sst2p, is also believed to play a role in this desensitization process, acting as the GTPase activating protein for Gα (Dohlmann et al., 1995).

In order for the cell to remain sensitive to the external pheromone cue, the cell actively breaks it down, in the case of α-factor the gene product BAR1 codes for a pepsin-like α-factor degrading protease, and in a similar manner SSL1 for a-factor.

**Pheromone inducible genes (PREs)**

When haploid yeast cells come into contact with the secreted pheromone of the opposite mating type the mating specific MAPK cascade is activated, resulting in a dramatic change both in the cell morphology and the transcription of pheromone inducible genes. These include those genes which are themselves responsible for pheromone production, such as the
pheromone precursors, $MFA1, MFA2$ and $MF\alpha 1, MF\alpha 2$. Other pheromone inducible genes are required for the response, $STE2$ and $STE3$ encoding the $MATa$ and $MAT\alpha$ cell type-specific receptor proteins, whilst others are required for the mating event per se. Including: $AGA1, AGA2$ and $AG\alpha 1$, the cell-surface agglutinins that are secreted extracellularly to allow for cell adhesion prior to cell fusion; $FUS1$, which is required for efficient cell fusion and is induced over 100-fold (Trueheart, J. et al., 1987; McCaffrey, M. et al., 1987); $FUS2$, which is required for both cell and nuclear fusion steps (Elion, E. A. et al., 1995); $FAR1$, which when activated (phosphorylated) causes arrest in G1 phase (Peter, M. and I. Herskowitz, 1994) and $KAR3$, which plays a role in nuclear fusion and is induced approximately 20-fold (Meluh, P. B. and M. D. Rose, 1990).

Many pheromone inducible genes are identifiable by the presence of a cis-acting DNA sequence, 5'-&(A)TGAAACA-3' (Kronstad et al., 1987), the pheromone response element, or PRE, located upstream of the promoter. $STE12$ is the transcriptional activator for the promoter containing PREs controlling the pheromone induced genes, it binds with equal affinity to DNA containing naturally occurring or synthetic PREs (Yuan, Y. L. and S. Fields, 1991). $STE12$ has been shown in vitro to become phosphorylated in response to pheromone (Bardwell, L. et al., 1994). PRE containing genes, including $FUS1$, demonstrate a low basal level of transcription when pheromone is absent, this is due to the intrinsic basal level of activity of the MAPK cascade. Experiments deleting the PREs upstream of $BAR1$ (Kronstad et al., 1987) and $FUS1$ (Hagen et al., 1991) resulted in complete loss of pheromone inducibility, particularly with $FUS1$ to the point that even the basal level of transcription was irradicated. This means that the PREs are the only upstream regions which are capable of activating $FUS1$ transcription.
Polarity establishment

In higher eukaryotic cells, including mammalian, cell polarity is responsible for establishing and maintaining functionally specialized domains in the plasma membrane and the cytoplasm (Drubin, D. G. and W. J. Nelson, 1996). The formation of such domains is critical for processes such as differentiation, localized membrane growth, immune response, cell migration, and vectorial ion transport of molecules across cell layers. Mammalian epithelial cells show a high degree of cellular asymmetry and have been used extensively as a model for cell polarity. Budding yeast cells are also considered to be an excellent model for cell polarity; although phylogenetically distant eukaryotes from epithelial cells, remarkably they both share a similarity of three sequential stages required for polarity establishment from a spatial cue (Drubin, D. G and W. J. Nelson, 1996). The three stages are hierarchical and consist of: marking a site and decoding the spatial cue; reinforcing the cue; and propagating the cue. A complex pattern of feedback regulation ensures that each stage is properly coordinated and ordered.

The budding program

Polarity establishment during the development of the growing bud involves a polarization of secretion and cell-surface growth, dependent on an underlying polarization of the cytoskeleton. The polarization of the cytoskeleton is dependent on a set of polarity-establishment proteins, including the small Rho-like guanosine triphosphate (GTP)-binding protein Cdc42p and its guanosine diphosphate (GDP)-GTP exchange factor Cdc24p, the Src homology 3 (SH3) domain-containing cell polarity establishment protein,
Bem1p, that associates with Cdc24p, a yeast formin, Bni1p, that links Cdc42p and the actin cytoskeleton, an actin associated protein Bud6p that participates in bipolar budding, the actin assembly related protein profilin and a yeast myosin Myo2p, required for vesicular transport (Adams et al., 1990; Amberg, D. et al., 1997; Bender, A. and J. Pringle, 1991; Chant, J. et al., 1991; Chant, J., 1994; Chenevert, J. et al., 1992; Chenevert, J. et al., 1994; Evangelista, M. et al., 1997; Govindan, B. et al., 1995; Johnson et al., 1990; Peterson et al., 1994; Sloat et al., 1981). The axes of polarization (i.e. bud sites) are typically selected in either of two patterns in a cell-type dependent manner. a or α haploid cells typically use the axial pattern, in which both mother and daughter cells bud adjacent to the preceding division site, whereas a/α diploid cells typically bud in the bipolar pattern, in which the daughter cell generally buds at the pole distal to the division site, and the mother cell can bud near either pole (Sloat et al., 1981; Streiblová, E. 1970). Both budding patterns depend upon a set of general site-selection proteins that comprise of a functional GTPase module. RSR1 (BUD1) encodes a Ras-related GTPase (Bender, A. and J. Pringle, 1989), whereas BUD2 and BUD5 encode a GTPase-activating protein and guanine-nucleotide-exchange factor, respectively, that act upon Rsr1p (Zheng, Y. et al., 1995). A null mutation in any of these three genes results in random budding irrespective of cell type. Rather than have a role only specific to the budding program, it has been noted that Rsr1p may also function in the fusion event in the mating program (Marsh, L., personal communication).

The mating program

The morphological changes that occur in yeast as they respond to pheromone during the mating response, is a second example of polarity establishment. As two haploid yeast cells of opposite mating type respond to
each others gradient of pheromone, they arrest their cell cycle in late G1 whilst continuing to grow. Each cell alters its pattern of growth from an isotropic manner, prior to pheromone response, to become highly polarized in response to pheromone as the cell concentrates new growth and membrane deposition to the developing mating projection, or shmoo. Such establishment of polarity is also witnessed during vegetative division when the yeast cell develops a new bud (Chant, J., 1994).

Polarized mating cells re-orient their actin cables to lie along the axis of the mating projection and localize patches of filamentous actin to the tip of the projection within the cell cortex (Chenevert, J. et al., 1994). Cytoplasmic microtubules are seen to extend from the spindle pole body (SPB), the yeast equivalent of the mammalian centriole, located adjacent to the nuclear membrane, and extend to a position at or near to the projection tip (Meluh, P.B. and M.D. Rose, 1990). The nucleus of each mating partner is positioned in or towards the neck of the mating projection with its spindle pole body facing the projection tip (Baba, M. et al., 1989; Byers, B. et al., 1981).

Components of the MAPK cascade have been implicated in the process of pheromone-induced polarized morphogenesis (Leeuw, T. et al., 1995). Bem1p has been shown to interact directly with Ste20p, Ste5p and actin. Mutants of Bem1p that show defects in pheromone-induced polarization interact with Ste5p and actin but not with Ste20p. Therefore Ste20p and Ste5p, through association with Bem1p, may convey spatial information that regulates the polarized rearrangement of the actin cytoskeleton during conjugation, (see Figure 1).

In response to a pheromone gradient the haploid cell continues to extend its shmoo until it comes into contact with the opposite mating type. Wild-type MATa cells saturated with exogenously added pheromone no longer distinguish a pheromone gradient, however they still develop a
mating projection. They do this by executing a default pathway whereby the development of an initial mating projection occurs near to their incipient bud site, and therefore they select a mate randomly (Dorer, R. et al., 1995). This means that under default conditions, the Bud1p GTPase cycle may control the formation of a mating projection.

**Bni1p**

Bni1p has been implicated in cytokinesis and cell polarization during both bud development and during pheromone response (Evangelista, M. et al., 1997; Jansen, R.-P. et al., 1996; Zahner, J. E. et al., 1996). Bni1p is a member of the formin family of proteins, which participate in cell polarization, cytokinesis, and vertebrate limb formation. Bni1p appears to provide a link between Cdc42p and the actin cytoskeleton during pheromone response (see Figure 1). All of the formins share two regions of sequence similarity, the (formin-homology) FH1 and the FH2 domains. Activated Cdc42p, in the GTP-bound state, forms a complex with Bni1p (Evangelista, M. et al., 1997). The FH1 domain of Bni1p binds actin and the actin-binding protein profilin. Bud6p, a protein that participates in bipolar budding and cellular morphogenesis shows a two-hybrid interaction with the COOH-terminal domain of Bni1p, and independently interacts with actin itself (Amberg, D. et al., 1997; Evangelista, M. et al., 1997). Rho1p-GTP, part of the family of five Rho-related GTPases, including Cdc42p, has also been demonstrated to bind the NH2-terminal portion of Bni1p (Kohno, H. et al., 1996). These results suggest that Bni1p may be part of a complex that is responsible for directing the assembly of actin filaments in response to Cdc42p signaling during polarized morphogenesis, i.e. during both budding and mating. Therefore common components appear to be utilized in polarization during both budding and mating in yeast.
Conjugation

Conjugation between yeast cells involves a series of well orchestrated cytoskeletal events, ultimately resulting in the fusion of the two nuclei and cytoplasms to generate a diploid cell. (1) Primarily the process begins with the initial contact, or agglutination, between the two mating projections. Agglutinins, encoded by the mating type specific structural genes AGα1, AGA1 and AGA2 are believed to be responsible for conferring the stickyness to the cells (Betz et al., 1979). (2) Secondly there follows a localized breakdown and remodelling of the cell walls of both partners and formation of a 'bridge', seen as a plate of electron-dense material, separating the two cell membranes (Kurjan, J., 1992). This defines the pre-zygote (Trueheart, J. et al., 1987). (3) The bridge separating the cell membranes of the pre-zygote breaks down, allowing the plasma membranes to fuse together to form one continuous membrane surrounding both partners. This defines the zygote. (4) Following membrane and cytoplasmic fusion the surrounding cell wall is remodelled and the nuclei are brought together to allow nuclear fusion (karyogamy) to take place.

The gene products of FUS1, FUS2 and FUS3 appear to play a fundamental role in the transition from the pre-zygote to the zygote stage during conjugation (Brizzio, V. et al., 1997; Chenevert, J. et al., 1994; Elion, E. A. et al., 1995; McCaffrey, M. et al., 1987; Trueheart, et al., 1987). Mutants in these genes are blocked at the pre-zygote stage in bilateral mating reactions.

Genes involved in nuclear fusion

The process of nuclear fusion (karyogamy) occurs through the active migration of the two mating partners nuclei towards one another. Correct positioning of the haploid nuclei is tubulin dependent (Page, B. and M.
Snyder, 1992), mediated through cytoplasmic microtubules connecting between the two SPBs (Kurjan, J., 1992). These microtubules shorten in length as the SPBs approach one another, with initial nuclear contact and fusion events occurring at the SPBs (Rose, M., 1991). Mutants in the kar (karyogamy) and cik (chromosome instability and karyogamy) genes show various defects in nuclear fusion and karyogamy (Meluh, P. and M. Rose, 1990; Page, B. and M. Snyder, 1992).

**Genes involved in cell fusion**

*FUS1* was originally isolated in two independent screens, one for pheromone inducible genes (McCaffrey, G. *et al.*, 1987) and the other by positional cloning to create mutants defective in mating (Trueheart, J. *et al.*, 1987). *FUS2*, a second pheromone-inducible gene (Elion, E. A. *et al.*, 1995), was isolated along with *FUS1*, and five other genes (*FUS3*-7) required for cell fusion on the basis of a block at the pre-zygote stage (McCaffrey, M. *et al.*, 1987; Trueheart, J. *et al.*, 1987).

*FUS1* encodes an 80 kDa protein product which is expressed over 100-fold in response to pheromone. *FUS1* encodes a 512 amino acid protein, with a short (71 amino acids) amino-terminal domain, separated by a stretch of 25 hydrophobic residues from a long carboxyl domain. The amino terminus consists of a majority of serine/threonine residues, O-glycosylated with short oligosaccharides made up of one to five mannose residues in length (Trueheart, J. and G. Fink, 1989). The amino terminus lies extracellularly, facing the periplasm. The short hydrophobic domain spans the plasma membrane once, followed by the much larger carboxyl domain on the intracellular side. (see Figure 2). The carboxyl domain of Fus1p (97-512 amino acids) possesses one src-homology 3 (SH3) domain near its terminus. SH3
Figure 2. The structure of Fus1p, showing the relative positions (in amino acids) of the amino membrane spanning domain, and carboxy SH3 binding motif and SH3 binding domain. The consensus sequence of the SH3 binding motif and SH3 binding domain are shown, aligned to the Fus1p sequence. The SH3 binding domain consensus sequence was calculated by comparing 64 separate SH3 binding domains from mammals, yeast and fruit fly proteins (Musacchio, A, et al., 1992). (Capital letters = residues with >85% conservation; Lower case = amino acids; % = >50% hydrophobic residues; # = >85% hydrophobic residues; — = >60% D + E).
Fuslp kplpltpons
Consensus KxxPxxP

SH3 binding motif

Fuslp 436 qlgktytviqdyeprltdeirislgekvki
Consensus ....##Alydy..-1tf.kg-..#
Fuslp lathtdgwclvekcntrkgtihvsvddkryl 496
Consensus #...g-Ww.a..&..G##Psnyv..#..
domains are found in a number of cytoskeletal and cytoplasmic proteins, including those involved in signal transduction pathways. The domains are believed to play a role in direct protein-protein interactions, SH3 domains are explained in further detail below. Fus1p also possesses a COOH-terminus SH3 binding motif (419-426 amino acids). This short motif is believed to bind specifically to SH3 binding domains (Musacchio, A. et al., 1992).

A FUS1-LacZ protein fusion localizes to the cell surface, specifically to the tip of the mating projection in a pheromone challenged haploid cell (Trueheart, J. et al., 1987). A FUS1-Green Fluorescent Protein (GFP) fusion localizes in an identical manner in response to pheromone (see Figure 3). As mentioned previously, the FUS1 promoter possesses tandem repeats of the pheromone response element (PRE), signifying that it is a pheromone responsive gene under the transcriptional control of the transcriptional activator STE12.

The FUS2 gene product has been shown to play a distinct role from Fus1p in the cell fusion event (Elion, E. A. et al., 1995). Fus2p is 73 kDa in size. Two pheromone response elements are found upstream of the FUS2 open reading frame, as expression of FUS2 is pheromone and Ste12p dependent. A common 5' 14-nucleotide stretch of identity (TATCTTTTTTCTTT) is shared between FUS1 and FUS2, located at equivalent distances from the START codons. The Fus2p sequence is unique, it does not possess an obvious transmembrane domain, and predictions of secondary structure suggest that it has an amphipathic α-helical structure containing coiled coil domains. Fus2p shows weak homology (~23% identity, 45% similarity) to yeast myosin-like protein, Mlp1 (Kolling, et al., 1993), mouse dystrophin (Bies et al., 1992) and a human kinesin-related protein (Yen et al., 1992). A fus1Δ x fus1Δ mating results in arrest as a pre-zygote, this is an example of a bilateral mating defect. fus2 mutants, on the other hand, show a defect in karyogamy as well as a
Figure 3. A FUS1-Green Fluorescent Protein fusion expressed in a wild-type MATa cell responding to an exogenous source of α-factor pheromone, observed under Panel A: differential interference contrast microscopy, Panel B: ultra-violet fluorescence microscopy. The FUS1-GFP fusion can be seen to localize to the tip of the mating projection in Panel B. (Courtesy of K. Blundell).
bilateral fusion defect. In fus2Δ x fus2Δ matings, zygotes display misalignment of parental nuclei, shown by a failure of the SPBs and associated microtubules to align correctly (Elion, E. et al., 1995). Fus1p and Fus2p localization overlaps in response to pheromone, however there is a difference between the two. Fus2p is seen as large punctate spots, resembling vesicles, positioned within the neck of the mating projection, at or near to the shmoo tip. Fus2p also does not appear to localize with the cell nucleus (Elion, E. et al., 1995).

Previously, it was suggested that FUS1 and FUS2 show a redundancy in function, based upon the available genetic evidence:
(1) In mating experiments, crossing either a fus1 or fus2 mutant to a wild-type partner of the opposite mating type results in only a partial reduction in mating efficiency (Trueheart, J. et al., 1987). Whereas, combinations of a double fus1 fus2 mutant crossed to a heterozygous fus1 or fus2 mutant reveals a significant reduction in mating efficiency; the most severe fusion defect seen by crossing a fus1 fus2 double mutant to a fus1 fus2 mutant (>1,000-fold more defective in mating than either single mutant), and
(2) multicopy FUS1 can suppress a fus2 mutants fusion defect, and vice versa.

This data suggests that both FUS1 and FUS2 are functioning together, or in a similar manner, to promote cell fusion. Fus1p may be directly involved in cell fusion, at the level of cell wall or plasma membrane breakdown and remodelling, for example, or may be acting more as a scaffold protein, associating with other proteins required for cell wall remodelling. The latter case is plausible due to the presence of one C-terminal SH3 domain, a domain known to interact with other cytoskeletal proteins.

FUS3 encodes a MAP kinase that has several functions in the pheromone signal transduction pathway (Elion, E. A. et al., 1990). Fus3p activates Ste12p and Far1p, an inhibitor of the Cdc28p-Cln complex necessary
to promote the pheromone-induced G1 arrest (Elion, E. A. et al., 1990; Fujimura, H. et al., 1990; Peter, M. et al., 1993; Tyers, M. and B. Futcher, 1993; Peter M. and I. Herskowitz, 1994; Herskowitz, I. 1995). The role of Fus3p in cell fusion remains unclear and may reflect either activation of a cell fusion gene via the pheromone response pathway or inactivation of a cell fusion inhibitor during the mitotic cell cycle (Brizzio, V. et al., 1996).

Recently, FUS5 and FUS8 were determined to be allelic to AXL1 and RAM1, respectively, two genes known to be required for biogenesis of a-factor (Brizzio, V. et al., 1996). This result suggests that a partial defect in a-factor production results in the fus phenotype, meaning that the level of pheromone is at least one important factor of the signal for prezygotes to initiate cell fusion.

**SH3 domains**

The SH3 domain was originally identified as a conserved sequence present in the N-terminal non-catalytic region of the nonreceptor class of mammalian src protein tyrosine kinases. The Src-kinases participate in signal transduction pathways that originate at the receptor tyrosine kinases, including the platelet derived growth factor receptor (PDGFR). Other proteins involved in these pathways also contain SH3 domains, including phospholipase Cγ, phosphatidylinositol-3-kinase (PI-3 kinase) and the p21ras GTPase activating protein (GAP) (Musacchio, A. et al., 1992). Subsequently SH3 domains have been found in many cytoplasmic and cytoskeletal related proteins, including those involved in signal transduction pathways, examples in *S. cerevisiae* include Fus1p (see Figure 3), Abp1, Bem1p, Rvs167p and Myo1p (Drubin, D. et al., 1988, 1990; McCaffrey, G. et al., 1987; Trueheart, J. et al., 1987). Recent studies on yeast actin using the yeast two-hybrid protein
interaction reporter system demonstrated an interaction between yeast actin and Fus1p, as well as a second SH3-domain containing protein, Rvs167p (Amberg, D. C. et al., 1995). By examining 35 actin mutants for two-hybrid interactions with Fus1p, Rvs167p, actin itself, profilin and Srv2p, they selected mutants which affected binding of some ligands rather than others. Using this 'differential interaction' pattern, they postulate that Fus1p and Rvs167p share a common binding site with actin, but cannot say if this occurs via the SH3 domains.

The SH3 protein domain is 55-75 amino acids in length. The domain has no fixed position in proteins, it appears that the position is not important for its function. The solution structure of two SH3 domain-ligand complexes has been obtained by nuclear magnetic resonance (Yu, H. et al., 1992; Feng, S. et al., 1994). The domain is composed of two short three-stranded anti-parallel β sheets packed together at right angles. Binding studies with proline-rich peptides revealed a hydrophobic ligand binding site on the SH3 domain surface that is associated with many of the conserved residues in SH3 domains. The most conserved and characteristic sequence in SH3 domains (ALYDY) defines one end of the binding site in Src (amino acids 88-92). Variation between different SH3 domains within the amino acids in the binding site may be responsible for their individual binding specificities to distinct peptides.

The RVS gene family

A mutant in the reduced viability on starvation (RVS) gene RVS161 arrests as a prezygote, in a similar manner to a fus1, fus2 and fus3 mutant (Dorer, R. et al., 1997; Favero, T., unpublished data). The mutation leads to a pleiotropic phenotype, RVS161 and its homologue RVS167 were originally

Rvsl6lp is similar to Rvs167p, and both are similar to amphiphysin, an acidic neuronal protein which was first identified in chicken synaptic vesicles (Lichte, B. *et al.*, 1992). Interestingly, amphiphysin was identified as an autoantigen of the rare autoimmune disease Stiff-Man syndrome (SMS) in female patients who were also afflicted with breast cancer (De Camilli, P. *et al.*, 1993). SMS is characterized by chronic rigidity of the body musculature with superimposed painful spasms. This may therefore provide a link between autoimmune disease and breast cancer.

*RVS161* is required for viability in stationary phase. An *rvs161* mutant shows a pleiotropic phenotype: the strain loses viability in response to nutrient (nitrogen, sulphur and carbon) starvation; it is sensitive to amino acid analogs, high salt concentration and does not grow on non-fermentable carbon sources. Like amphiphysin deficient mutants, *rvs161* mutants also show an endocytosis defect (Crouzet, M. *et al.*, 1991). *RVS161* and *RVS167* are essential for bud site selection; *rvs161* and *rvs167* mutants show a wild-type axial budding pattern as haploids under standard conditions, but show a disrupted random budding pattern as diploids (Bauer, F. *et al.*, 1993; Durrens, P. *et al.*, 1995; Sivadon, P. *et al.*, 1995).

*RVS161* has recently been shown to be pheromone inducible (Erdman, S. and M. Snyder, personal communication), thus implicating a role in the pheromone induced cell fusion event. The mutant defect and the fact that *RVS161* is pheromone inducible, suggests that Rvsl6lp may be involved in actin-based endocytosis secretion, possibly targetting specific proteins to the mating projection that are required for membrane remodelling or cell fusion.

Rvsl6lp is a 30 kDa protein that shares significant homology over its entire length with the Rvs167p NH$_2$-terminal domain. Rvs167p is a 53 kDa
protein that can be divided into three specific domains. The NH₂-terminal
domain, which shows homology with Rvs161p, contains two potential
cAMP-dependent phosphorylation sites (Bauer, F. et al., 1993). The C-terminal
domain contains one conserved SH3 domain sequence, preceded by a 130
amino acid domain rich in glycine, proline and alanine (GPA-rich). Rvs167p,
yeast Abp1p, non-filamentous myosin I of Dictyostelium discoideum and
Acanthamoeba castellanii, and pp80/85, a pp60SRC substrate, all possess a GPA
rich sequence preceding an SH3 domain. The charges on the GPA-rich
sequences differ within this group: positively charged in myosin I; negatively
charged in Abp1p and pp80/85; without any charge in Rvs167p. This may
have functional significance for these proteins. It has been suggested that
proteins containing a GPA-rich domain and SH3 domain function in cortical
actin cytoskeleton organization (Drubin, D. G. et al., 1990; Zot, H. G. et al.,
1992). Like rvs161, rvs167 mutants also demonstrate an endocytosis defect,
suggesting that they have a similar or related role.

Rvs167p has been demonstrated to show an in vivo interaction with
actin using the two-hybrid reporter system (Amberg, D. C. et al., 1995).

The sequence homology between Rvs161p and Rvs167p, their similar
pleiotropic mutant defects, and homology with the human auto-antigen
amphiphysin suggest that the proteins play a similar, if not concerted, role in
control of the actin cytoskeleton. In fact, Rvs167p has been shown to interact
with Rvs161p in vivo via the two-hybrid system (Crouzet, M. et al., 1995;
Ritchie S., M. Evangelista, and C. Boone., unpublished results). This strongly
argues that both RVS161 and RVS167 act together to mediate actin based
endocytosis, in association with the cortical actin cytoskeleton, a function that
is important in the pheromone induced cell fusion event.
Chitin synthase 5

Chitin synthase 5 (CHS5) is another gene that has been implicated to be involved in pheromone induced cell fusion (Favero, T., unpublished results; Santos, B. et al., 1997).

Chitin is a structural polysaccharide that is an essential component of the yeast cell wall. In *S. cerevisiae*, chitin is found mostly in the bud scar, a craterlike structure present on the surface of the mother cell following cell separation (Aacon, J. S. et al., 1969; Cabib, E. and B. Bowers, 1971).

Formation of the bud scar during the budding cycle occurs in several steps (Cabib, E. et al., 1974). Concomitant with bud emergence, a chitin ring is laid down at the base of the growing bud; chitin continues to be deposited as the bud grows to the size of the mother cell. The complex responsible for this synthesis, chitin synthase III (CSIII), requires the action of several genes. These are CHS3 (Bulawa, C. E. et al., 1992; Cabib, E., 1994; Pammer, M. et al., 1992; Valdivieso, M. H. et al., 1991), CHS4, CHS5 and CHS6 (Bulawa, C. E. et al., 1992; Roncero, C. et al., 1988). CHS3 is expected to code for the catalytic component of CSIII. CHS4, CHS5, and CHS6 have all been implicated both in resistance to the Calcofluor white stain and in chitin synthesis mediated specifically by CSIII activity, but exact functions still remain to be established for them (Cid, V. J. et al., 1995).

During cytokinesis the deposition of chitin changes from a ring to a centripetal fashion in the furrow of the invaginating plasma membrane. This results in the formation of a thin electron-translucent disk structure, termed the primary septum (Cabib, E. et al., 1974). The primary septum is synthesized by the action of a separate chitin synthase activity, chitin synthase II (CSII), encoded by the CHS2 gene (Silverman, S. J. et al., 1988). The two cells separate asymmetrically, with the majority of the chitin remaining in the mother cell.
as a bud scar. A periplasmic chitinase and the function of chitin synthase I (CSI), encoded by CHS1, is required for cell separation. CSI acts to repress the chitinase activity in the manner of a safety mechanism during the final process of cell separation (Cabib, E. et al., 1992).

Chitin is also present in small quantity uniformly throughout the cell wall of the mother cell; CSIII is responsible for this synthesis (Roncero, C. et al., 1988; Shaw, J. A. et al., 1991).

Chitin synthesis in *S. cerevisiae* is not specific only to the mitotic cycle. During the process of mating projection (shmoo) formation, in response to an extracellular pheromone cue, chitin is synthesized and deposited mainly at the subapical portion of the shmoo (Shekman, R. et al., 1979). CSIII is wholly responsible for chitin synthesis in the growing shmoo (Roncero, C. et al., 1988; Shaw, J. A. et al., 1991), remarkably, although CHS1 transcription is highly induced by exposure to pheromone, CHS3 transcription is not. At least one of the MAPK genes, *FUS3* or *KSS1*, of the pheromone response MAPK cascade is required for the twofold increase in chitin synthesis in response to α-factor (Santos, B. et al., 1997).

CHS3 plays a role in one more stage of the *S. cerevisiae* life cycle, sporulation. It lays down chitosan, a derivative of chitin in the ascospore walls (Briza, P. et al., 1988).

As mentioned above, little is known of the true function for CHS4, CHS5, and CH6 gene products. Recently, CHS5 has been discovered to have an important role during the cell fusion step of mating (Santos, B. et al., 1997). CHS5 was cloned by complementation of the Calcofluor resistance phenotype of a previously isolated chs5 mutant. Chs5p is 73.6 kDa in size, shows no significant similarity to any protein present in the current databases, and bears no relation to any of the other proteins encoded by the chitin synthase genes.
Mutating CHS5 results in resistance to Calcofluor and reduces the amount of cell wall chitin to 25% of that observed in a wild-type cell. A chs5Δ disruption strain forms a mating projection which is much broader and rounder than wild-type. This phenotype is very similar to the mating projections formed in a chs3Δ strain, which indicates that chitin may play a role in the mating event.

Mating two isogenic chs5Δ strains together results in only 15% of the number of diploids formed compared with mating two isogenic wild-type strains; for further comparison a chs3Δ x chs3Δ mating results in 47% of the diploids compared to wild-type, all in the absence of Calcofluor. Examining the cell pairs (bilobated zygotes) in the chs5Δ x chs5Δ mating revealed that the majority were binucleate and failed to degrade their cell walls at the point of fusion. This defect was not observed at the level of nuclear fusion (Santos, B. et al., 1997), but was at the level of the prezygote, before the cell wall degrades and plasma membrane fuses (Trueheart, J. et al., 1987). Therefore the defect is similar to the fus1, fus2, fus3 and rvs161 mating defects.

Interestingly, the chs5Δ mating defect is independent of the level of chitin as the defect is more severe than that seen in a chs3Δ mutant strain, which shows a lower level of chitin in response to α-factor than a chs5Δ strain. The chs5Δ mating defect is similar to the fusion defect associated with fus1, fus2 and fus3 mutants (Elion, E. A. et al., 1990, 1995; Trueheart, J. et al., 1987).

Partial suppression of the chs5 mating defect occurred if FUS1 or FUS2 was expressed on a centromeric or multicopy plasmid. However, the fus1 fus2 mating defect was not suppressed by expressing CHS5, therefore CHS5 and FUS1 and/or FUS2 do not function in partially redundant pathways.
Over expression of \( FUS3 \) did not alleviate the \( chs5A \) mating defect, therefore \( CHS5 \) appears to act downstream of \( FUS3 \) and upstream of \( FUS1 \) and/or \( FUS2 \) (Santos, B. et al., 1997).

Affirming the role of \( CHS5 \) in the cell fusion event, our laboratory isolated \( CHS5 \) in a synthetic-sterile genetic screen to identify genes required for cell fusion (Favero, T., unpublished data). Using an \( sst2A \) strain, which exacerbates the mating defect associated with a fusion mutant in a bilateral cross, \( FUS1, FUS2, FUS3, CHS5, \) and \( SPA2 \) were isolated amongst others (Boone, C. et al., 1996).

Chs5p shows a limited homology to neurofilament proteins and myosin heavy chains, but no precise motifs have been identified (Santos, B. et al., 1997). Chs5p localizes in cytoplasmic patches and may be responsible for targeting Chs3p to polarized growth sites during vegetative growth and in response to \( \alpha \)-factor (Santos, B. and M. Snyder, 1997). Myo2p is also required for the correct targeting of Chs3p, therefore presenting a molecular mechanism for this transport.

A model whereby Chs5p may be associated with transport vesicles that deliver not only Chs3p, but other cell wall modifying or cell fusion specific proteins, such as Fus1p and Fus2p to the membrane, has been suggested. This may represent another pathway of actin-based secretion, analogous to that proposed for Rvs161p and Rvs167p.

**The Two-Hybrid System**

The yeast two-hybrid system (Fields, S. and O-K. Song, 1989) is a simple genetic assay that allows detection of protein-protein interactions directly. The assay can be used in determining protein interactions between independent proteins or between components of protein complexes. Determining
networks of interactions between components of complexes provides what has been termed as a 'protein linkage map' (Evangelista, C. *et al.*, 1996), which furthers our understanding of the function and control of a protein complex.

The two-hybrid system exploits the ability of a pair of interacting proteins to bring a transcriptional activation domain into close proximity with a DNA-binding site that regulates the expression of an adjacent reporter gene, such as the gene encoding the yeast auxotrophic marker *LEU2* or the *E. coli* reporter gene *lacZ*. The reporter genes and appropriate upstream activation sequences (UAS) are present on the chromosome (targetted via homologous recombination) of the yeast strain used in the two-hybrid experiments. Hybrids are constructed encoding a DNA binding domain fused to a protein X and an activation domain fused to a protein Y. The DNA binding domain targets the hybrid protein to its binding site; however, because most proteins do not possess an activation domain, the hybrid will not activate expression of the reporter gene(s). Similarly, the hybrid protein that contains the activation domain will not activate expression of the reporter gene as it cannot bind the UAS of the gene. However, when both hybrids are present in close proximity, due to the protein-protein interaction between X and Y, the activation domain is tethered to the UAS and so expression of the reporter gene(s) occurs. Different levels of expression of the reporter gene *lacZ* is commonly seen for different protein-protein interactions assayed, this is not indicative of the 'strength' of interaction between the proteins and therefore cannot be attributed to the degree of binding.

One of the most powerful uses of the two-hybrid system is the ability to screen cDNA and genomic DNA libraries for proteins that interact with a protein of interest, the 'bait'. This assay is carried out using two reporter genes in order to limit the number of false positives. False positives may be a result of proteins that possess cryptic activation domains that activate expression of
the reporter gene(s) without interacting with the 'bait' protein at all. These false positives have to be screened by testing for reporter gene expression in the absence of the 'bait' hybrid gene.

The protein-protein interactions in the two-hybrid assay occur in the nucleus of the yeast cell, however, many protein interactions that normally occur in the cytoplasm of the cell have been shown to take place in the nucleus. The fact that a positive interaction is not shown by the assay does not mean that the proteins do not interact in their native environment. Not all proteins may be functionally active due to conformational changes, for example, that occur in the chemically distinct environment of the nucleus, or if they require a post-translational modification that does not normally occur in yeast. Certain proteins that cannot fold correctly or be imported into the nucleus may be unsuitable in a two-hybrid assay and therefore be unrepresented in a cDNA or genomic library screen.

Another powerful use of the two-hybrid assay is in the delineation of the domains of interaction between proteins by making suitable deletions in the genes encoding proteins X and Y and assaying for reporter gene expression. Subsequently, residues can be identified within the interacting domains of X and Y that are critical for the interaction to occur. One problem with this method is that certain deletions or mutations in one hybrid gene may increase transcriptional activation significantly, which may be as a result of exposing residues in a shorter domain that are not available in the intact protein, or because a smaller protein or domain simply works better in the system.

Protein-protein interactions detected using the two-hybrid system, as in other methods, may be the result of artefactual signals, therefore these interactions should be confirmed biochemically utilising glutathione-S-transferase tagged versions of these proteins in an in vitro assay, for example.
MATERIALS AND METHODS

Strains and media

Yeast strains used in this study are listed in Table I. Yeast strains were grown on rich media that contained 2% Bacto-peptone (Difco Laboratories, Detroit, MI), 1% Bacto-yeast extract (Difco), 0.012% adenine sulfate and 2% glucose (YEPD+Adenine) or 5% galactose (YEPGAL+Adenine), solid media contained 2% agar (BDH). Transformants were selected on synthetic (S) medium that contained 0.7% Yeast Nitrogen Base without amino acids (Difco), supplemented with the required amino acids (Laboratory Manual, Yeast Genetics Course, Cold Spring Harbor) and either 2% glucose (Sigma), SD medium, 2% galactose (Sigma), SGAL medium, or 2% raffinose (Sigma), SRAF medium. In mating experiments, diploids were selected on SD minimal medium without any addition of amino acids.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3031A</td>
<td>MAT a leu2-3, 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15</td>
<td>Boone laboratory</td>
</tr>
<tr>
<td>W3031B</td>
<td>MAT α leu2-3, 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15</td>
<td></td>
</tr>
<tr>
<td>SY2014</td>
<td>MAT α sst2Δ</td>
<td></td>
</tr>
<tr>
<td>SY2575</td>
<td>MAT a/α his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,</td>
<td>G. Sprague</td>
</tr>
<tr>
<td></td>
<td>-112 trp1-1 ade2-1 can1-100 (homozygous for all markers)</td>
<td></td>
</tr>
<tr>
<td>SY2625</td>
<td>MAT a bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,</td>
<td>G. Sprague</td>
</tr>
<tr>
<td></td>
<td>-112 trp1-1 ade2-1 can1-100</td>
<td></td>
</tr>
<tr>
<td>Y62</td>
<td>MAT a lys1 cry1</td>
<td>I. Herskowitz</td>
</tr>
<tr>
<td>Y63</td>
<td>MAT α lys1</td>
<td>I. Herskowitz</td>
</tr>
<tr>
<td>Y66</td>
<td>MAT a far1-c lys1 (enfeebled mater see Chenevert et al., 1994)</td>
<td>J. Chenevert</td>
</tr>
<tr>
<td>Y67</td>
<td>MAT α far1-c lys1</td>
<td>J. Chenevert</td>
</tr>
<tr>
<td>Y106</td>
<td>MAT a fus1Δ sst2::LEU2 lys2::GAL-SST2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ</td>
<td>T. Favero</td>
</tr>
<tr>
<td></td>
<td>ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100</td>
<td></td>
</tr>
<tr>
<td>Y389</td>
<td>MAT a fus1Δ sst2Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112</td>
<td>T. Favero</td>
</tr>
<tr>
<td></td>
<td>lys2 trp1-1 ade2-1 can1-100</td>
<td></td>
</tr>
<tr>
<td>Y498</td>
<td>MAT α pRS306::fus1Δ sst2Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>lys2 trp1-1 ade2-1 can1-100</td>
<td></td>
</tr>
<tr>
<td>Y499</td>
<td>MAT a sst2Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 lys2</td>
<td>Boone laboratory</td>
</tr>
<tr>
<td></td>
<td>trp1-1 ade2-1 can1-100</td>
<td></td>
</tr>
</tbody>
</table>

28
Table I. Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y550</td>
<td>MAT a GAL-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ trp1-901 leu2-3,-112 ura3-52 his3-200 gal4Δ gal80Δ</td>
</tr>
<tr>
<td>Y596</td>
<td>MAT a lexAop-Leu2 ura3::URA3-lexAop-LacZ, his3 trp1 ura3-52 leu2 GAL+</td>
</tr>
<tr>
<td>Y688</td>
<td>MAT a/α bni1Δ::LEU2/BN11 trp1/TRP1 LYS2/lys2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1::ura3-1 leu2-3,-112 ade2-1 can1-100</td>
</tr>
<tr>
<td>Y704</td>
<td>MAT a lexAop-HIS3 lexAop-lacZ sst1Δ his3 trp1 ura3-52 leu2</td>
</tr>
<tr>
<td>Y742</td>
<td>MAT a ade8Δ::MET3-a2 sst2Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 ade2-1 can1-100</td>
</tr>
<tr>
<td>Y790</td>
<td>MAT a bni1Δ::URA3 full length KO trp1 LYS2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 ade2-1 can1-100</td>
</tr>
<tr>
<td>Y822</td>
<td>MAT a bni1Δ::LEU2 full length KOrp1 LYS2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 ade2-1 can1-100</td>
</tr>
</tbody>
</table>
| Y900   | MAT a URA+ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ leu2-3,-112 ade2-1 can1-100 (p1309 TRP+)

This study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Traits</th>
</tr>
</thead>
</table>
| Y901   | MAT α URA+ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ leu2-3,-112 ade2-1 can1-100 (V26/URA3+)

This study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Traits</th>
</tr>
</thead>
</table>
| Y999   | MAT a exs1Δ::TRP bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 ade2-1 can1-100 (p1289 TRP+)

This study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1005</td>
<td>MAT a exs1Δ::TRP bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 ade2-1 can1-100</td>
</tr>
</tbody>
</table>

This study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1006</td>
<td>MAT a bnr1Δ::URA3 trp1 LYS2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 ade2-1 can1-100</td>
</tr>
</tbody>
</table>

This study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1007</td>
<td>MAT α bnr1::URA3 trp1 LYS2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 ade2-1 can1-100</td>
</tr>
</tbody>
</table>

This study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1058</td>
<td>MAT a bnr1::URA3 bar1Δ::LEU2 trp1 LYS2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 ade2-1 can1-100</td>
</tr>
</tbody>
</table>

This study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Traits</th>
</tr>
</thead>
</table>
| Y1097  | MAT a ste7Δ::ADE2 lexAop-LacZ ura3: (URA3, lexAop-LacZ), leu2 his3 trp1 ade2 lys2 gal80 GAL4

This study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Traits</th>
</tr>
</thead>
</table>
| Y1098  | MAT a ste11Δ::ADE2 lexAop-LacZ ura3: (URA3, lexAop-LacZ), leu2 his3 trp1 ade2 lys2 gal80 GAL4

This study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Traits</th>
</tr>
</thead>
</table>
| Y1141  | MAT a bnr1Δ::TRP1 trp1 LYS2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 ade2-1 can1-100 (p1289 TRP+)

This study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1142</td>
<td>MAT α bnr1Δ::TRP1 trp1 LYS2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 ade2-1 can1-100</td>
</tr>
</tbody>
</table>

This study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1158</td>
<td>MAT a bnr1::LEU2 bnr1::URA3 bar1Δ trp1 LYS2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 ade2-1 can1-100</td>
</tr>
</tbody>
</table>

PCR checked

This study
Plasmid construction

The E. coli strains MC1061 and DH5α were used for the construction and propagation of plasmids. The five different fragments of FUS1 were generated by PCR (Daugherty, B. L. et al., 1991) using the oligonucleotide primers shown in Table II.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUS1 5' (1)</td>
<td>GGATCCGTTAACATGGTAGCAA CAATAATGCAGACGACA</td>
<td>5' N-terminus of FUS1 including the ATG (from amino acid 1), with 5' BamHI site.</td>
</tr>
<tr>
<td>FUS1 5' (97)</td>
<td>GGATCCGTTAACATGAAAAGGA ATTCGGTGTCCATTTCA</td>
<td>5' N-terminus of FUS1 excluding membrane spanning domain (from amino acid 97), with 5' BamHI site and ATG.</td>
</tr>
<tr>
<td>FUS1 5' (312)</td>
<td>GGATCCGTTAACATGCCTGACG AACGATCGCCCATC</td>
<td>5' of FUS1 excluding N-terminus and membrane spanning domain, with 5' BamHI site and ATG.</td>
</tr>
<tr>
<td>FUS1 5' (410)</td>
<td>GGATCCGTTAACATGTATCAGCA TTTACAACATTITGGTGACG</td>
<td>5' including SH3 domain of FUS1 (from amino acid 410), with 5' BamHI site and ATG.</td>
</tr>
<tr>
<td>FUS1 3' (512)</td>
<td>GAGCTCTCAGTCGTATTTTGG AGACAGTCACC</td>
<td>3' C-terminus of FUS1, including STOP codon at amino acid 512, with SstI site.</td>
</tr>
<tr>
<td>FUS1 3' (435)</td>
<td>GAGCTCTCAGACGCTAGCCTCT CCATTATATTIGGA</td>
<td>3' C-terminus excluding the SH3 domain, including STOP at amino acid 435, with SstI site.</td>
</tr>
<tr>
<td>YIP91</td>
<td>CTGCAGGGTTAACATGGACTCTT CC</td>
<td>5' N-terminus of BNR1 including the ATG (from amino acid 1), with 5' XhoI site.</td>
</tr>
<tr>
<td>YIP92</td>
<td>CTGCAGTTTCAGATTTTCAATT TCCITT</td>
<td>3' of BNR1 including amino acid (581) and PstI site.</td>
</tr>
<tr>
<td>YIP93</td>
<td>CTGCAGTTGAAAGCACAACAG AAA</td>
<td>5' of BNR1 from amino acid (580), including PstI site.</td>
</tr>
<tr>
<td>YIP94</td>
<td>GCCGCCCCGTATATATTTTGAA TATC</td>
<td>3' C-terminus of BNR1 including STOP codon at amino acid (1376), with NotI site.</td>
</tr>
<tr>
<td>URA3PCRko</td>
<td>GTGCTCCTTCCTCTGGCTTCTTCTTC TCC</td>
<td>3' of URA3 from amino acid (32), to check URA3 knockout strain.</td>
</tr>
<tr>
<td>URA3upPCR</td>
<td>ATTCGAGTGAAACACAGGAA</td>
<td>5' from 716bp upstream of URA3 to check URA3 knockout strain.</td>
</tr>
<tr>
<td>PSTItoNOT1</td>
<td>AGCGGCCGCTTCCA</td>
<td>Converts a PstI site to a NotI site.</td>
</tr>
</tbody>
</table>

Table II. PCR primers and oligonucleotides
In order to generate Bluescript KS+ vectors containing \textit{BamHI/SstI} clones of the five fragments of \textit{FUS1}, the oligonucleotides listed in Table II were used as primers to PCR from a YEp24 plasmid containing \textit{FUS1} genomic DNA as a template, p31 (see Table III). The five fragments of \textit{FUS1} generated, and the combinations of oligonucleotide primers used were: 1. (1-512aa) \textit{FUS1} 5' 1/FUS1 3' 512; 2. (97-512aa) \textit{FUS1} 5' 97/FUS1 3' 512; 3. (1-473aa) \textit{FUS1} 5' 1/FUS1 3' 473; 4. (97-473aa) \textit{FUS1} 5' 97/FUS1 3' 473; 5. (312-512aa) \textit{FUS1} 5' 312/FUS1 3' 512. All of the PCR products were gel purified (GeneCleanII) and cloned as blunt-ended fragments into the \textit{EcoRV} site of Bluescript KS+ vector, thus creating the following plasmids: p511 (1-512aa); p543 (97-512aa); p323 (1-435aa); p544 (97-435aa); and p545 (312-512aa).

To generate the \textit{GAL1} driven versions of the \textit{FUS1} clones, each clone was isolated as a \textit{BamHI/SstI} fragment from the appropriate Bluescript KS+ vector and subcloned into the \textit{BamHI/SstI} sites of p113, a pRS316 derived vector containing the \textit{GAL1} promoter with a modified pRS316 downstream polylinker, thus creating the following plasmids: p333 (1-512aa); p396 (97-512aa); p412 (1-435aa); p413 (97-435aa); and p397 (312-512aa), see Table III.

To generate the two-hybrid DNA-binding domain (DBD) and transcriptional-activation domain (AD) fusions of selected \textit{FUS1} clones; p543, p544 and p545 were digested with \textit{BamHI/XhoI} and the \textit{FUS1} containing fragments, including the \textit{XhoI} from the Bluescript KS+ polylinker were gel purified (GeneCleanII). The gel purified fragments were ligated to the \textit{BamHI/SalI} sites of DBD pGBDU-C1, p538, (reading in the GGA frame), generating p546, p547 and p581, see Table III. p546 and p547 were sequenced and confirmed to be correct (data not shown). Two of the fragments were ligated to a derivative of the LexA DBD pBTM116NLS (reading in the GGA frame), V60, (Durfee, T. \textit{et al.}, 1993), generating p1336 and p1108, see Table III; and also ligated to the \textit{BamHI/SalI} sites of the AD plasmid pGAD-C1, p1599,
(James, P. et al., 1996), generating p564 and p582, see Table III. The same fragments were also ligated to the BamHI/Xhol sites of a derivative of pEG202 (GGA frame), p736, (Gyuris, J. et al., 1993), generating p1002, p1004 and p1003, see Table III.

Two-hybrid libraries were obtained as AD fusions present in the plasmids pJG4-5 (Gyuris, J. et al., 1993), pGAD-C1 (James, P. et al., 1996), and pACT (Durfee, T. et al., 1993).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Details</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>p5</td>
<td>YEp24 plasmid</td>
<td>P. Hieter</td>
</tr>
<tr>
<td>p31</td>
<td>FUS1 (genomic copy), pSL324</td>
<td>G. Sprague</td>
</tr>
<tr>
<td>p113</td>
<td>pRS316::GAL1. promoter (with Kpn1-EcoRV of poly linker removed via digestion with Kpn1 and EcoRI, klenow reaction, and ligation</td>
<td>C. Boone</td>
</tr>
<tr>
<td>p264</td>
<td>YEp24::FUS1 isolated from multicopy screen</td>
<td>This study</td>
</tr>
<tr>
<td>p304</td>
<td>YEp24::FUS2 isolated from multicopy screen</td>
<td>This study</td>
</tr>
<tr>
<td>p323</td>
<td>KS+::FUS1 (1-435aa). Ligated 1.3kb PCR fragment to EcoRV of KS+ oligo #1284/1286</td>
<td>This study</td>
</tr>
<tr>
<td>p333</td>
<td>GAL1::FUS1 (1-512aa). p511 cut with BamHI/XbaI and ligated 1.4 kb gel purified fragment to p113 cut with BamHI/XbaI. Sequenced: SR44 (in reverse)</td>
<td>This study</td>
</tr>
<tr>
<td>p396</td>
<td>GAL1::FUS1 (97-512aa). p543 cut with BamHI/SstI and ligated gel purified fragment to p113 cut with BamHI SstI. Sequenced: SR43 (correct)</td>
<td>This study</td>
</tr>
<tr>
<td>p397</td>
<td>GAL1::FUS1 (312-512aa). p545 cut with BamHI/SstI and ligated gel purified fragment to p113 cut with BamHI SstI. Sequenced: SR 45 (correct)</td>
<td>This study</td>
</tr>
<tr>
<td>p412</td>
<td>GAL1::FUS1 (1-435aa). p323 cut with BamHI/SstI and ligated gel purified fragment to p113 cut with BamHI SstI. Sequenced: SR46 (in reverse)</td>
<td>This study</td>
</tr>
<tr>
<td>p413</td>
<td>GAL1::FUS1 (97-435aa) p544 cut with BamHI/SstI and ligated gel purified fragment to p113 cut with BamHI SstI. Sequenced: SR47 (correct)</td>
<td>This study</td>
</tr>
<tr>
<td>p439</td>
<td>pGAD424::BEM1 Bem1p (1-551)</td>
<td>R. Lacoeque</td>
</tr>
<tr>
<td>p455</td>
<td>pEG202 vector ADH prom, LexA DBD, HIS marker two hybrid plasmid</td>
<td>E. Golemis</td>
</tr>
<tr>
<td>p471</td>
<td>YEp24::MFA1 isolated from multicopy screen</td>
<td>This study</td>
</tr>
<tr>
<td>p511</td>
<td>pNoTA/T7::FUS1 (1-512aa). Ligated 1.4kb PCR fragment to MCS of 2.7kb vector using the PCR-Script ligation kit. Sequenced: SR35 (correct)</td>
<td>This study</td>
</tr>
<tr>
<td>p538</td>
<td>pGBDU-C1/NotI. pGBDU-C1 cut with Pst I and ligated PSTItoNOTI oligo to change Pst I site to Not I site [GGA of BamHI is in frame]</td>
<td>This study</td>
</tr>
<tr>
<td>p543</td>
<td>KS+::FUS1 (97-512aa). Ligated 1.25kb PCR fragment to EcoRV site of KS+</td>
<td>This study</td>
</tr>
<tr>
<td>p544</td>
<td>KS+::FUS1 (97-435aa). Ligated 1.02kb PCR fragment to EcoRV site of KS+</td>
<td>This study</td>
</tr>
<tr>
<td>p545</td>
<td>KS+::FUS1 (312-512aa). Ligated 1.0kb PCR fragment to EcoRV site of KS+</td>
<td>This study</td>
</tr>
<tr>
<td>p546</td>
<td>pGBDU-C1::FUS1 (97-512aa). p543 cut with BamHI/Xhol and ligated gel purified fragment to pGBDU-C1 cut with BamHI/Sall. Sequenced: SR33 (correct)</td>
<td>This study</td>
</tr>
<tr>
<td>p547</td>
<td>pGBDU-C1::FUS1 (312-512aa). p545 cut with BamHI/Xhol and ligated gel purified fragment to pGBDU-C1 cut with BamHI/Sall. Sequenced: SR34 (correct)</td>
<td>This study</td>
</tr>
</tbody>
</table>
p563 pGAD::FUS1 (1-435aa). p412 cut with BamHI/Xhol and ligated gel purified fragment to pGAD-C1 cut with BamHI/Xhol

p564 pGAD::FUS1 (97-512aa). p396 cut with BamHI/Xhol and ligated gel purified fragment to pGAD-C1(GGA) cut with BamHI/Xhol

p581 pGBDU-C1::FUS1 (97-435aa). p544 cut with BamHI/Xhol and ligated gel purified fragment to pGBDU-C1 cut with BamHI/SalI

p582 pGAD::FUS1 (312-512). p545 cut with BamHI/Xhol and ligated gel purified fragment to pGAD-C1 cut with BamHI/Xhol

p624 pAIP14 (2m, LEU2, Profilin, 7-126)

p625 pAIP18 (2m, LEU2, Srv2p/CAP, 5-526)

p626 pAIP70 (2m, LEU2, Actin, 24-375)

p627 pAIP143 (2m., LEU2, Rvs167p, 1-549)

p628 pAIP161 (2m, LEU2, FUS1p, 97-512)

p649 pGBDU-C1::FUS1 (1-435aa). p323 cut with BamHI/Xhol and ligated gel purified fragment to pGBDU-C1 cut with BamHI/Sall

p688 pGAD424::BUD6 (Bud6p C-terminal 311 amino acids)

p736 pEG202 (GGA frame). oligonucleotide linkers GGAA/GGAB ligated to p55 cut with EcoRI, to switch codon reading frame to GGA in BamHI site

p892 pACT2, ADH::Gal4AD DNA activation domain vector

p972 GAL1-RVS161 (EIci36II to NotI). p267 cut with Eci36II and ligated New England Biolabs NotI 12-mer in to site

p974 pGAD::RVS161 p208 cut with BglII/NotI and ligated into pGAD424-GGA cut with BamHI/NotI.

p1001 PEG202::FUS1 (1-435aa). p323 cut with BamHI/Xhol and gel purified fragment ligated to p736 cut with BamHI/Xhol

p1002 PEG202::FUS1 (97-512aa). p543 cut with BamHI/Xhol and gel purified fragment ligated to p736 cut with BamHI/Xhol

p1003 PEG202::FUS1 (97-435aa). p544 cut with BamHI/Xhol and gel purified fragment ligated to p736 cut with BamHI/Xhol

p1004 PEG202::FUS1 (312-512aa). p545 cut with BamHI/Xhol and gel purified fragment ligated to p736 cut with BamHI/Xhol

p1081 CY2651 (pSL2174) GAL4::FUS3 (pMA424 DBD based) 2 micron pADH1 HIS5, can use in Brent system

p1082 CY2652 (pSL2175) GAL4::FUS3 (pGAD424 AD based) 2 micron pADH1 LEU2, can use in J. James system

p1108 pBTM116 NLS (V45)::FUS1 (97-435aa). p544 cut with BamHI/Xhol and gel purified fragment ligated to V45 cut with BamHI/Xhol

p1111 GAL1p::GAL4 AD (pGLG-5') two-hybrid vector

p1117 pBTM116 NLS (V45)::FUS1 (1-435aa). p323 cut with BamHI/Xhol and gel purified fragment ligated to V45 cut with BamHI/Xhol

p1118 pEG202::CHS5. p535 cut with BglII/NotI and gel purified fragment ligated to p736 cut with BamHI/NotI

p1120 pACT::ACT1 . Actin in pAct vector recovered from Catch & Release 2-hybrid screen (9-1)

p1121 pACT::ACT1 . Actin in pAct vector recovered from Catch & Release 2-hybrid screen (12-1)

p1129 pGLG-5::BNR1. pGLG-5 containing BNR1 (297-789aa) from library search with p1002 [PEG202::FUS1(97-512aa)]. Sequence: SR22

p1130 pGLG-5::YNL054W. pGLG-5 containing YNL054W (aa) from library search with p1002 [PEG202::FUS1(97-512aa)]. Sequence: SR21

p1134 pGLG-5::CHS5 . p535 cut with BglII/NotI and ligated CHS5 to p738(pGLG-5-GGA) cut with BamHI/NotI

p1139 pBTM116 NLS::RVS161 . p208 cut with BamHI/NotI and ligated gel purified RVS161 fragment to V46 cut with BamHI Notl
PCR-Script SK+::YNL054W (690-1167aa KpnI - NotI). Ligated 1441bp PCR fragment to MCS of 2.8kb plasmid using the PCR cloning protocol.

KS+::BNRl. p1129 cut with EcoRI to isolate 1.2kb BNRl fragment and ligated to KS+ cut with EcoRI, BNRl is present C to N term orientation in respect to BamHl/Xhol

KS+::YN1054W. p1130 cut with EcoRl to isolate 1180bp gel purified fragment and ligated to KS+ cut with EcoRl, YNL054W is present N to C in respect to BamHl/Xhol

KS+(minus polylinker)::YNL054W. p1183 cut with EcoRl and ligated to p1647 cut with EcoRl to make knockout.

KS+(minus polylinker)::YLR117C. p1521 cut with EcoRl and ligated to p1523 cut with EcoRl to make knockout.

pACT2::RVS167, two-hybrid library clone (33-483aa)

KS+(minus polylinker)::YNL054W+Bglll. p1269 cut with Ec113611 and ligated in Bglll linker)

KS+(minus polylinker)::YLR117C+Bglll. p1274 cut with Ec113611 and ligated in Bglll linker

KS+(minus polylinker)::YN1054wA::URA3. p1294 cut with BglII and ligated in Bglll URA3 fragment (V19), cut with EcoRl to target KS+(minus polylinker)::YN1075W. p1295 cut with BglII and ligated in Bglll URA3 fragment (V19), cut with EcoRl to target

pBTM116NLS::FUSI (97-512aa). p543 cut with BamHl/Xhol and gel purified fragment ligated to V60 cut with BamHl/Xhol

pBTM116NLS::FUSI (1-435aa). p323 cut with BamHl/Xhol and gel purified fragment ligated to V60 cut with BamHl/Xhol

pGBDU-C1::FUSI (97-512aa)Notl. p546 cut with Pstl and ligated 'PstI to Notl' linker

pGBDU-C3/Notl::RVS161. p208 cut with BamHl/Notl and ligated gel purified fragment to p573 cut with BamHl/Notl

pGBDU-C1::FUSI (1-435aa)Notl. p649 cut with Pstl and ligated 'PstI to Notl' linker

PCR-Script SK+:::FUSI SH3 domain (410-512aa). Ligated 315bp PCR fragment to MCS of 2.8kb vector using the PCR cloning protocol.

pACT1 promoter::GFP::RVS161. p208 cut with BamHl/Notl and ligated gel purified fragment to p1335 cut with BamHl/Notl (Cen URA3 vector)

KS+:::FUSI SH3 domain (410-512aa). p1365 cut with BamHl/Sstl and ligated gel purified fragment to KS+ cut with BamHl/Sstl

KS+:::FUSI SH3 domain (410-512aa) + Notl site. cut p1405 with Eco136II and ligated the New England Biolabs Notl 12-mer

pGAD2F LEU2 2µ, pADH1, GAL4 Activation Domain

pSTE4 AD LEU2 2µ, pADH1, GAL4 Activation Domain, STE4

pSTE11 AD LEU2 2µ, pADH1, GAL4 Activation Domain, STE11

pKSS1 AD LEU2 2µ, pADH1, GAL4 Activation Domain, KSS1

pSTE7 AD LEU2 2µ, pADH1, GAL4 Activation Domain, STE7

pSTE5 AD LEU2 2µ, pADH1, GAL4 Activation Domain, STE5

pSTE11 K444R AD LEU2 2µ, pADH1, GAL4 Activation Domain, STE11K444R catalytically dead

pFUS3 AD LEU2 2µ, pADH1, GAL4 Activation Domain, FUS3

pSTE20 AD LEU2 2µ pADH1, GAL4 Activation Domain, STE20

S. Givan

L. Pratt

This study
p1469 SK+::BNR1 (1-587aa). PCR of BNR1 using primers Yip91/Yip92 with Expand system. (1743bp) cloned into SK+ vector using Stratagene kit

p1470 SK+::BNR1 (587-1375aa) PCR of BNR1 using primers Yip93/Yip94 with Expand system. (2384bp) cloned into SK+ vector using Stratagene kit STE12 AD LEU2 2μ, STE12 in pGAD2

p1491 pRS316:: FUS1 + promoter-GFP, p1444 Xho/Nru + p1371 Xho/Nru

p1516 pGAD3F-GPA1 2μ, LEU2 AD-GPA1

p1518 pGAD1F-SST2 2μ, LEU2 AD-SST2

p1521 pJG4-5::YLR117C. pJG4-5 containing YLR117C from library search with p1002 [PEG202::FUS1(97-512aa)]. Sequence: SR28

p1522 pJG4-5:: BUD2. pJG4-5 containing BUD2 from library search with p1002 [PEG202::FUS1(97-512aa)]. Sequence: SR24

p1523 pJG4-5::YNL075W. pJG4-5 containing YNL075W from library search with p1002 [PEG202::FUS1(97-512aa)]. Sequence: SR27

p1524 KS+::BNR1 (1-587aa). p1469 cut with Xhol/PstI and ligated to KS+ cut with Xhol/PstI

This study

p1527 KS+::BNR1Δ::URA3. p1185 cut with BgIII and ligated in BgIII URA3 fragment cut with EcoRI to target

This study

p1530 pBTM116NLS:: FUS1 (410-512aa). p1414 cut with BamHI/NotI and FUS1(410-512aa) ligated to V59 cut with BamHI/NotI

This study

p1531 KS+::BNR1 (587-1375aa). p1470 cut with PstI/NotI and ligated BNR1 fragment to KS+ cut with PstI/NotI

This study

p1534 SK+::BNR1 FH1 domain (2263-2850bp). PCR product of FH1UP/FH1DWN cloned into SK+ vector, in the ATC frame

This study

p1576 pEG202::BNR1 FH1 domain (2263-2850bp). p1534 cut with BamHI/NotI and gel purified fragment ligated to p455 cut with BamHI/NotI

This study

p1577 pACT2::BNR1 FH1 domain (2263-2850bp). p1534 cut with BamHI/NotI and gel purified fragment ligated to p892 cut with BamHI/NotI

This study

p1578 pBTM116NLS::BNR1 FH1 domain (2263-2850bp). p1534 cut with BamHI/NotI and gel purified fragment ligated to V46 cut with BamHI/NotI

This study

p1586 pGAD424:: PE2A V47 BamHI/NotI + p1567 BamHI/NotI

M. Evangelista

p1595 KS+::BNR1 (1-1375aa) p1531 cut with PstI/NotI and gel purified 2.3Kb fragment (BNR1 587-1375aa), ligated to p1524 cut with Xhol/PstI

This study

p1599 pGAD-C1 vector, in the GGA frame.

P. James

p1600 pGAD-C2 vector, in the ATC frame.

P. James

p1607 pGBDU-C1(NotI)::CHS5. p408 cut with BgIII/NotI and ligated gel purified fragment to p538 digested with BamHI/NotI

This study

p1608 pBTM116NLS::CHS5. p408 cut with BgIII/NotI and CHS5 gel purified fragment ligated into V59 (GGA frame) cut with BamHI/NotI

This study

p1609 pGAD-C1 NotI. p1599 cut with PstI and ligated in oligo PstIto NotI

This study

p1610 pGAD-C2 NotI. p1600 cut with PstI and ligated in oligo PstIto NotI

This study

p1616 stellA::ADE2 2.2 kb BgIII ADE2 fragment inserted into BgIII site. Cut with Xhol/BamHI to target.

J. Printen

p1617 ste7A::ADE2 2.5 kb ADE2 fragment of pAZ810 inserted into BgIII site. Cut with Xhol/PstI or with HindIII to target.

J. Printen

p1626 pGAD:: RVS161. p208 cut with BamHI/NotI and ligated gel purified fragment to p1611 cut with BamHI/NotI.

This study

p1647 KS+(minus polylinker)::EcoRI linker. V5 (KS+) cut with PvuII and polylinker removed, EcoRI linker added to lose PvuII site.

This study

p1648 TOPO::YNL075w. PCR product of YNL075w from Vent PCR with YNL075up/YNL075dwn cloned into TOPO vector using the kit. YNL075w has BamHI and NotI flanking sites, in the ATC frame.

This study
Table III. Plasmids

**Yeast methods and transformations**

Standard yeast genetic manipulations were carried out as described (Sherman *et al.*, 1986). Yeast transformations were performed by the lithium acetate method (Ito, H. *et al.*, 1983; Hirsch, J. P. *et al.*, 1993). Yeast plasmid isolations were performed as described, (Guthrie and Fink, 1993).

**Molecular biological techniques**

Standard molecular biological techniques were used for construction of plasmids, DNA sequencing and PCR (Sambrook, J., E. F. Fritsch and T. Maniatis, CSHL Press, 1989). Plasmids used in this study are listed in Table III. DNA sequences were determined using an ALFred DNA sequencer (Pharmacia Biotech Inc., Sweden) by University Core DNA Services, University of Calgary, Calgary, AB. PCRs were performed using a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT). Cloning of PCR products was carried out directly by gel-purifying (and ligating to the EcoRV site of Bluescript KS+ vector (Stratagene) or by using the PCR-Script® (Stratagene) or TOPO® (Invitrogen) PCR cloning kits.
PCR check of \emph{bnr1\Delta::URA3} gene disruption strains

Genomic DNA was extracted from \emph{bnr1\Delta BNI1} (Y1006, Y1007) and \emph{bnr1\Delta bni1\Delta} (Y940, Y941), \emph{MAT\alpha} and \emph{MAT\alpha} versions, respectively, gene disruption strains. PCR was carried out on this genomic DNA in order to confirm that \emph{BNR1} was successfully disrupted with the \emph{URA3} gene. Vent PCR reactions using primers Yip91 and URA3PCRkocheck were carried out for each strain; as a control the same PCR reactions were carried out with no primers added.

Multicopy suppression screen of \emph{fus1\Delta}

The strain Y106 (see Table I) was transformed with a yeast genomic library in the multicopy plasmid YEp24 (Carlson, M. and D. Botstein, 1982) and plated out onto SD-URA media. An efficiency of approximately $2 \times 10^3$ transformants per plate was routinely obtained. In order to screen for multicopy suppressors of the \emph{fus1\Delta sst2\Delta} phenotype, the transformation plates were replica plated to a fresh lawn of the W303 background wild-type tester strain Y66 (see Table I) on YEPD+Ade media. The cells were allowed to mate for two and a half to three hours at 30°C before being replica plated to SD media to select for diploids. Colonies showing suppression of the mating defect were visualised on the SD plates as patches of white diploids.

Yeast two-hybrid method

Three two-hybrid "systems" were used in this study, in each case a plasmid containing a gene fused to the DNA binding domain (DBD), pGBDU-C, pEG202 or pBTM116NLS, was transformed into strain Y550, Y596 or Y704.
respectively. The resultant transformants were transformed with a plasmid containing a specific gene or a library of genomic DNA fragments fused to the transcriptional-activation domain (AD) pGAD, pJG4-5 or pACT, respectively.

Selection of library positives was as follows: the pGBDU-C/pGAD system was made on SD media lacking uracil, leucine and adenine; the pEG202/pJG4-5 system on media lacking histidine, tryptophan and leucine; the pBTM116NLS/pACT system on media lacking tryptophan, leucine and histidine, in the presence of 10mM adenosine triazitol (ATZ). Quick LacZ assays were performed by dissolving one loopful of cells of each transformant or library positive in 100μl of ONPG+LacZ buffer (see materials), adding 5μl 20% (w/v) sarkosyl and 5μl formaldehyde, vortexing briefly then incubating at 37°C. For quantitative assay of β-galactosidase activity, cells of each pGBDU-C/pGAD or pBTM116NLS/pACT transformant/library positive were cultured in SD medium lacking uracil and leucine or tryptophan and leucine, respectively. For quantitative assay of transformants/library positives from pEG202/pJG4-5 the cells were grown to mid-log phase in SRAF medium lacking histidine and tryptophan, galactose was added to a concentration of 2%, and growth continued for 4 hours before assaying for β-galactosidase activity. All transformants/library positives were assayed for β-galactosidase activity as described, (Hagen, D. C. et al., 1991).

**Pheromone response assay**

A bar1Δ strain was grown overnight at 30°C in 5ml YEPD+Ade. 200 μl was transferred into 5 ml fresh YEPD+Ade and cultured for 2 hours at 30°C. One ml of culture was transferred into a fresh tube, adding 5 μl of α-factor (1/25 dilution of 1mg/ml soln) (Sigma), and grown at 30°C. After 1, 2, 3, and 4 hours, 250 μl of culture was centrifuged to form a pellet, @ 6000 rpm, 1 min.
The supernatant was discarded and 100 µl of fixative (3.7% formaldehyde, 0.15 M NaCl) added. These were stored at 4°C until viewed on a conventional light microscope. Mating projections were compared against a control of wild-type cells that were assayed under the same conditions (Guthrie and Fink, 1993).

**G1 arrest plate assay**

Approximately one-half of an inoculating loop of the yeast strain of interest was mixed in 1ml of YEPD+Ade, mixed and transferred 100 µl into a fresh tube of 900 µl of YEPD+Ade. 300 µl of this dilution was spread onto a YEPD+Ade plate and allowed to dry. 5 µl of α-factor (1/25 dilution and 1/50 dilution of 1mg/ml soln) (Sigma), were spotted onto the plate and incubated at 30°C. A control plate of a wild-type bar1Δ strain (SY2625) was treated in the same manner. G1 arrest was shown by the formation of a zone of no growth surrounding the spot of α-factor. Reference (Guthrie and Fink, 1993).
RESULTS

Multicopy suppressor hunt in a fus1Δ sst2Δ background

Previously, a mutant hunt was carried out in our laboratory to screen for genes involved in the mating response of *S. cerevisiae* (Boone, C. *et al.*, 1996; Favero, T., unpublished data; Dorer, R. *et al.*, 1997). This approach involved using a strain background possessing a null mutation in the synthetic sterility gene *SST2*, a pheromone desensitization factor responsible for the recovery of cells arrested in response to mating pheromone (Dohlman, H. G. *et al.*, 1995). This allowed for isolation of mutants that showed a unilateral mating defect. The screen assayed for mutants showing a decreased level of mating relative to the level shown by wild-type controls. Complementation of the mutants revealed a number of genes previously implicated in the mating response, including *FUS1*, *FUS2*, and *FUS3*, as well as genes which were not previously known to have a role in mating, including *BNII*, *SPA2*, *CHS5*, and *RVS161*. All of these genes, important in the cell fusion event, demonstrate a bilateral mating defect, i.e. in a fus1 x fus1 mating.

The fusion gene *FUS1* was selected for further analysis as it plays a definitive part in the cell fusion event; it is actively transcribed in response to pheromone; and its protein product possesses a C-terminal SH3 (src homology-3 domain), believed to be responsible for protein-protein interactions between cytoskeletal components.

An sst2Δ background exacerbates the mating defect associated with a fus1Δ strain when mating to a wild-type or compromised mating tester strain. This allowed for the use of a multicopy suppressor approach to attempt
to define proteins interacting on a genetic level with Fus1p, a so-called multicopy suppressor search.

The strain Y106 (see Methods, Table I) was transformed with a multi-copy YEp24 based yeast genomic DNA library in order to screen for suppressors of the mating defect associated with the fus1Δsst2Δ mutation (see Methods). Colonies showing suppression of the mating defect were visualized on the SD plates as patches of white diploids. Over 5 x 10⁵ colonies were screened in total. Potential suppressors were streaked out for single colonies on SD-URA to select for a single YEp24 clone. In order to demonstrate plasmid dependency of the suppression, suppressors were streaked for single colonies on 5-fluoro-orotic acid (FOA) medium (see Methods). Those which lost the YEp24 cDNA clone with a resultant loss of suppression of the fus1Δsst2Δ defect were retained for further analysis.

Plasmid DNA was extracted from the suppressors (see Methods) and then reintroduced, by transformation, back into strain Y106 in order to re-confirm suppression. Thirty-three positive suppressors were retained and analyzed by restriction analysis in order to compare the genomic DNA clones isolated (data not shown). Five YEp24 genomic DNA clones appeared to be independent from one another, based upon the restriction analyses. Sequencing quality DNA was prepared for each suppressor and the YEp24 genomic DNA clones were sequenced using the YEp24upper and YEp24lower primers (see Methods). Sequences were compared with the complete S. cerevisiae genomic nucleotide sequence using the Saccharomyces Genome Database (SGD) BLAST program. All five of the sequences were of five independent genes, FUS1, FUS2, MFA1, MFA2 and SST2. Further restriction analysis was carried out to confirm that all twenty-eight remaining suppressors encoded one of the five genes (see Table IV). Qualitative mating assays were performed as described (see Materials and Methods) using Y106.
Table IV. Multicopy suppressors of the Y106 fus1Δssi2 mating defect. Gene name, the number of times isolated from the screen and the relative level of suppression of the mating defect compared to wild-type (**=wild-type level of mating).

Table V. Interactions of Fus1p (97-512aa), p1336, with Rvs167p (p627), Fus1p (p628), Srv2p (p625), Rvs161p (p974), profilin (p624), actin (p626) and pACT2 (p892). Interaction of Rvs167 (p627) and Rvs161p (p1139), Rvs161p (p1139) and Fus1p (p628) are included as additional controls. Interactions were detected with the two-hybrid system. Numbers in parentheses indicate the expressed amino acids. Data (Miller units) are means ± SD of three independent experiments.
<table>
<thead>
<tr>
<th>Number of times isolated</th>
<th>Level of Suppression</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>***</td>
<td>MFA1</td>
</tr>
<tr>
<td>4</td>
<td>**</td>
<td>MFA2</td>
</tr>
<tr>
<td>8</td>
<td>***</td>
<td>SST2</td>
</tr>
<tr>
<td>13</td>
<td>***</td>
<td>FUS1</td>
</tr>
<tr>
<td>2</td>
<td>***</td>
<td>FUS2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gal4p-AD fusions</th>
<th>LexA-DBD fusions</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>p627 Rvs167p</td>
<td>Fuslp (97-512)</td>
<td>7.80 ± 0.76</td>
</tr>
<tr>
<td>p628 Fuslp (97-512)</td>
<td>Fuslp (97-512)</td>
<td>38.76 ± 1.65</td>
</tr>
<tr>
<td>p625 Srv2p</td>
<td>Fuslp (97-512)</td>
<td>0.43 ± 0.15</td>
</tr>
<tr>
<td>p892 pACT2</td>
<td>Fuslp (97-512)</td>
<td>0.71 ± 0.09</td>
</tr>
<tr>
<td>p624 profilin</td>
<td>Fuslp (97-512)</td>
<td>0.33 ± 0.05</td>
</tr>
<tr>
<td>p626 actin</td>
<td>Fuslp (97-512)</td>
<td>0.67 ± 0.17</td>
</tr>
<tr>
<td>p974 Rvs161p</td>
<td>Fuslp (97-512)</td>
<td>0.54 ± 0.07</td>
</tr>
<tr>
<td>p627 Rvs167p</td>
<td>Rvs161p (p1139)</td>
<td>64.83 ± 4.53</td>
</tr>
</tbody>
</table>
transformed with the five multicopy suppressors (see Figure 4). This result demonstrates that genes suppressing a bilateral mating defect can be isolated in a unilateral manner using an \textit{sst2}\textDelta background. I isolated two \textit{FUS} genes and the \textit{a}-factor structural genes, \textit{MFA1} and \textit{MFA2}. Recently it has been shown that the cell fusion defect associated with the \textit{a}-factor specific \textit{axl1} and \textit{ram1} could be suppressed by overexpression of \textit{a}-factor (Brizzio, et al., 1996). A mutant form of the \textit{a}-factor ABC-transporter gene \textit{STE6} has also been implicated in a cell fusion defect.\textit{ste6(cef)} accumulates as prezygotes during mating, indicating that the level of \textit{a}-factor is important for mating and cell fusion (Elia, L. and L. Marsh, 1996).

\textbf{Two-Hybrid experiments with Fus1p and proteins involved in reorganization of the actin cytoskeleton}

It has previously been demonstrated that a two-hybrid interaction occurs between the cytoplasmic domain of Fus1p (97-512aa) and a number of actin point mutants (Amberg, D. C. \textit{et al.}, 1995). The actin associated protein, profilin and the actin-associated endocytosis vesicle gene product, \textit{Rvs167p} were shown to possess a similar 'differential interaction pattern' as Fus1p when examined for a two-hybrid interaction against the actin point mutants. This presented the question of whether Fus1p, \textit{Rvs167p} and profilin interact with one another in a complex, and therefore may contribute to actin based secretion that is important in the cell fusion event.

The PCR product of \textit{FUS1} (97-512aa), encoding the cytoplasmic domain, was cloned into the two-hybrid LexA DNA binding domain (DBD) vector, pBTM116NLS (p1336) (see Methods). p1336 was tested for interaction in two-hybrid experiments against the DNA activation domain (AD) vector, pACT2(p892), containing profilin(p624), actin(p626), and actin binding
proteins, RVS167(p627), FUS1(p628), CAP/SRV2(p625), and RVS161(p974) and no insert as a negative control. The results of these experiments are shown in Table V. A strong interaction was noted between Fus1p and Rvs167p, and between Fus1p and Fus1p (see Table V). A strong interaction was shown between Rvs161p and Rvs167p. No interaction was noted between Fus1p and actin, contrary to the previous experiments (Amberg, D. C. et al., 1995). Fus1p did not interact with profilin, Srv2p, Rvs161p or with the empty vector.

In order to determine the specific regions of Fus1p responsible for these interactions, three of the previous PCR versions of FUS1 were cloned into the DBD pBTM116NLS and tested against Rvs167p, and Fus1p (see Methods). These were: 1) excluding the membrane spanning domain and the SH3 domain (97-435aa), p1108; 2) excluding the membrane spanning domain and N-terminus (312-512aa), p547; and 3) the SH3 domain and C-terminus alone (410-512aa), p1530 (see Figure 2.). However, none of these DBD constructs demonstrated a positive interaction with the AD Rvs167p, Fus3p or Fus1p (data not shown). This may be due to incorrect folding of these truncated proteins or the constructs being incorrect. In order to check the fidelity of the PCR products, all of the three FUS1 fragment constructs were sequenced using a primer reading from the GAL1 promoter, the original vector containing the PCR fragments. All three sequences were correct, and were expected to be transcribed in the correct reading frame (data not shown). Therefore I assumed that either no interaction was taking place or that the protein products were incorrectly folded, and so functionally inactive.
Figure 4. Qualitative mating assay of Y106 fus1Δsst2Δ transformed with: A) YEpl4 vector (p5), B) YEpl4::FUS1(p264), C) YEpl4::FUS2(p304) and D) YEpl4::MF11(p471), with E) wild-type (W3031A), mated to Y63 for four hours before replica plating to SD medium.

Figure 5. Qualitative mating assay of A)fus1Δ sst2Δ(Y106), B) wild-type (W3031A), C)rus167Δ rus161Δ(Y1096), D)rus167Δ fus1Δ(Y1117) E)rus161Δ fus1Δ(Y736) and F) rus161Δ(Y1094), mated to an enfeebled mater strain (Y66) on YEPD+Ade medium for four hours before replica plating to SD medium.
**Genetic experiments with RVS167**

RVS161 has previously been demonstrated to have a role in mating, an *rus161Δsst2Δ* shows a mating defect when mated to a wild-type strain of the opposite mating type (Boone, C. *et al.*, 1996; Dorer, R. *et al.*, 1997).

As RVS161 shows considerable homology to RVS167 and has been shown to interact directly with RVS167 in a two-hybrid assay (Crouzet, M. *et al.*, 1995; Pratt L. and S. Ritchie, this thesis), it was logical to examine if RVS167 plays a role in the cell fusion event during mating. A gene disruption of RVS167 was made in an *rus161Δ, fus1Δ*, and wild-type background. A gene disruption of RVS161 in a wild-type and a *fus1Δ* background was included for comparison. The *rus167Δrus161Δ* (Y1096), *rus167Δfus1Δ* (Y1117) *rus161Δfus1Δ* (Y736) and *rus161Δ* (Y1094) strains were used in qualitative mating assays against an enfeebled mater strain (Y66), (see Methods and Figure 5.). The *rus161Δfus1Δ* (Y736) (panel E) mating defect was severe, equivalent to that seen for *fus1Δsst2Δ* (Y106) (panel A). The *rus167Δfus1Δ* (Y1117) (panel D) strain appeared to have a weak mating defect compared with the wild-type (panel B) and *rus161Δ* (Y1094) (panel F) strains, and mated much better than the *rus167Δrus161Δ* (Y1096) (panel C) strain. This result further confirms the role of RVS161 in mating. Therefore, RVS167 is important for cell mating, but appears to play a more subtle role than RVS161.
Two-Hybrid experiments with Fus1p and the MAPK cascade pathway components.

Fus1p and Fus2p are important components in mating, and it is believed that they may themselves be regulated by components of the MAPK pheromone response pathway (Boone, C., personal communication). The fact that a FUS3 mutant shows a mating defect provides additional evidence for this hypothesis. This led to the question of whether Fus1p interacts with Fus3p or any of the other components of the pheromone response pathway. DNA AD fusions of the pheromone response pathway components, STE4, STE5, STE7, STE11, STE11-K444R, STE20, STE12, FUS3, KSS1 and other mating specific components FUS2, BEM1, BUD6, PEA2 and ACT1 were transformed into strain Y704 which had first been transformed with the DNA BD (p1336) pBTM116NLS::FUS1(97-512aa). Quantitative LacZ assays were performed for each combination (see Methods), the results are presented in Table VI. The strongest LacZ activity was seen with Kss1p, Fus3p, and Fus1p itself. A strong interaction was seen with Ste5p, the MAPK pathway scaffold protein. A weaker interaction was seen with Ste11p (MEKK), which was lost when the catalytically-inactive mutant Ste11p-K444R was tested. Interestingly, Fus2p failed to show any interaction with Fus1p, however in the reciprocal experiment, with the DBD pBTM116NLS::FUS2 versus p564, pGAD::FUS1 (97-512), a strong level of interaction was noted (Evangelista, M., personal communication). Bem1p, Bud6p, Pea2p and actin failed to show a positive interaction with Fus1p.

In order to determine whether the MAPK pathway components interact independently of one another with Fus1p, the same interactions were measured in both a ste7Δ and a ste11Δ background (Table VI). The results indicate that the protein interactions between Fus1p and Ste5p and Ste11p are
Table VI. Interactions of Fus1p (97-512aa), p1336, with the MAPK pheromone response pathway components and other cytoskeletal proteins in wild-type (Y704), ste7Δ(Y1097), and ste11Δ(Y1098) backgrounds. Interactions were detected with the two-hybrid system (see Methods). Data (Miller units) are means ± SD of three independent experiments. (++ = positive quick LacZ assay, — = negative quick LacZ assay.)
<table>
<thead>
<tr>
<th>Gal4p-AD fusions</th>
<th>LexA DBD fusion</th>
<th>β-Galactosidase activity (Miller units ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild-Type</td>
</tr>
<tr>
<td>p1435 Fus3p</td>
<td>p1336 Fus1p</td>
<td>48.32 ± 5.31</td>
</tr>
<tr>
<td>p1426 Kss1p</td>
<td>p1336 Fus1p</td>
<td>68.98 ± 6.71</td>
</tr>
<tr>
<td>p1432 Ste5p</td>
<td>p1336 Fus1p</td>
<td>19.21 ± 0.43</td>
</tr>
<tr>
<td>p1423 Ste11p</td>
<td>p1336 Fus1p</td>
<td>4.77 ± 1.13</td>
</tr>
<tr>
<td>p1434 Ste11p K444R</td>
<td>p1336 Fus1p</td>
<td>0.85 ± 0.12</td>
</tr>
<tr>
<td>p1428 Ste7p</td>
<td>p1336 Fus1p</td>
<td>2.53 ± 0.04</td>
</tr>
<tr>
<td>p1438 Ste20p</td>
<td>p1336 Fus1p</td>
<td>1.28 ± 0.13</td>
</tr>
<tr>
<td>p628 Fus1p</td>
<td>p1336 Fus1p</td>
<td>38.76 ± 1.65</td>
</tr>
<tr>
<td>p1487 Ste12p</td>
<td>p1336 Fus1p</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>p1586 Pea2p</td>
<td>p1336 Fus1p</td>
<td>1.47 ± 0.13</td>
</tr>
<tr>
<td>p1571 Fus2p</td>
<td>p1336 Fus1p</td>
<td>1.19 ± 0.26</td>
</tr>
<tr>
<td>p1120 Actín</td>
<td>p1336 Fus1p</td>
<td>0.67 ± 0.16</td>
</tr>
<tr>
<td>p1124 Actin</td>
<td>p1336 Fus1p</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>p439 Bem1p</td>
<td>p1336 Fus1p</td>
<td>0.89 ± 0.15</td>
</tr>
<tr>
<td>p688 Bud6p</td>
<td>p1336 Fus1p</td>
<td>1.00 ± 0.16</td>
</tr>
<tr>
<td>p892 pACT2</td>
<td>p1336 Fus1p</td>
<td>0.71 ± 0.10</td>
</tr>
</tbody>
</table>
all lost when either Ste7p or Ste11p are no longer expressed. Therefore either Ste5p, Ste11p and Ste7p may all be lost as a complex or a pheromone inducible gene that possesses a cryptic transcriptional activation domain can associate with Fus1p, and may indirectly activate transcription. This result demonstrates a strong level of interaction between Fus1p and Fus3p and Kss1p, respectively. This provides a putative link between the cell fusion machinery and the MAPK pheromone response cascade.

**Two-Hybrid Library search with Fus1p as bait**

As the two-hybrid system demonstrated interactions between Fus1p and specific cytoskeletal proteins and components of the pheromone response pathway, the next step was to identify additional proteins that may form part of a putative complex with Fus1p. A yeast two-hybrid library screen was chosen to do this. A yeast two-hybrid cDNA library of LexA activation-domain fusions was screened against the Fus1p DNA binding-domain fusion (97-512aa) pEG202::FUS1 (p1002) in strain Y596 (see Methods). Protein interactions were scored by the ability of the transformed cells to express the LEU2 gene under the control of a LexA-responsive promoter. Protein interactions were further confirmed by the ability to produce β-galactosidase from a lacZ gene fused separately to a LexA-responsive promoter. Final confirmation of protein interaction was made after isolating the positive LexA activation-domain fusions and re-transforming them back into Y596 transformed with pEG202::FUS1, and detecting β-galactosidase activity. In order to completely saturate the library, over $6 \times 10^5$ transformants were screened; this covered the library more than two times. Five independent clones were recovered from the library, including two known genes and three unknown genes. BNR1 (YIL159w) was isolated independently three times,
BUD2 was isolated only once, YNL054w was isolated independently three times, YNL075w and YLR117c were both isolated only once (see Figure 6.). Quantitative LacZ assays were performed on these positives (see Methods and Table VII).

BNR1 (standing for BNI1 related) is a homolog of BNI1, the yeast formin that links Cdc42p and the actin cytoskeleton during polarized morphogenesis (Evangelista, M. et al., Science, 1997). Like BNI1, BNR1 possesses both an FH1 (formin homology 1) and an FH2 (formin homology 2) domain, the FH1 domain of BNR1 has recently been shown to interact with profilin (Imamura, H. et al., 1997). The two-hybrid clone we isolated encodes most of the amino terminus of Bnrlp (297-789aa), including 35 amino acids of the FH1 domain (see Figure 6.). This stretch of amino acids includes the polyproline rich sequence "PQLPPPPPPPPPPPLP". Proline rich sequences have been implicated as regions to which SH3 domains bind.

BUD2 is the GAP (GTPase-activating-protein) for RSR1/BUD1, the Ras-like element considered to be essential for axial and bipolar budding. A mutation in bud1, bud2 or bud5 causes a random budding phenotype in cells, irrespective of mating type (Drubin, D. et al., 1991).

The predicted protein encoded by YNL054w had no known homologs when examined in BLASTp searches (Altschul, S. F. et al., 1990) against the current proteins present in the databases, it does however possess a putative single COOH-terminal trans-membrane domain based upon the predicted proteins hydrophobicity plot (Figure 7). A copy of the cytoplasmic domain containing the YNL054w ORF was obtained as a cDNA clone.

YNL075w shows significant sequence similarity to a putative Caenorhabditis elegans ORF, ZK795c (56% identity over 74 residues). A sequence alignment of the predicted proteins encoded by YNL075w and
**Figure 6.** A schematic diagram showing the genes isolated from a two-hybrid library screen with Fus1p (97-512aa). *BNRI, BUD2, YNL054w, YNL075w* and *YLR117c* are depicted with the shaded box corresponding to the fragment isolated from the library. The region corresponding to the formin-homology 1 (FH1) domain is depicted for *BNRI*. Numbers correspond to amino acids in the predicted translated protein products.
BNR1

BUD2

YNL054w

YNL075w

YLR117c
ZK795c is shown in Figure 8. BLASTp results show that this sequence appears to be strongly conserved in mammals too. A mouse cDNA clone (W63859) demonstrates 59% identity with Ynl075wp over 44 residues, and a human cDNA clone (H61261) demonstrated 57% identity with Ynl075wp over 106 residues (data not shown). Although the gene appears to be highly conserved, no indication to what the function of the putative protein can be obtained based upon these results.

YLR117c shows sequence similarity to a Drosophila melanogaster gene, crooked neck (crn) (42% identity over 191 residues), (Zhang, K., et al., 1991). This is based upon a repeated sequence with similarity to a TPR (tetratricopeptide repeat) found in multiple copies in the crn gene. TPRs have previously been noted in a family of fungal genes, including three yeast cell cycle genes, CDC16 and CDC23 of S. cerevisiae (Hartwell 1976; Pringle and Hartwell 1981; Icho and Wickner 1987) and nuc2+ of Schizosaccharomyces pombe (Hirano et al. 1988, 1990). All three genes are required for progression through the G2/M stage of the cell cycle. Loss of zygotic expression of crn leads to defects in proliferation of brain neuroblasts and loss of some neuronal lineages in the developing D. melanogaster embryo (Zhang, K., et al., 1991). A sequence alignment of Ylr117cp against the crooked neck protein product is shown in Figure 9.

**Gene disruptions of BNR1, YNL054w, YNL075w and YLR117c**

Gene disruptions of BNR1, YNL054w, YNL075w and YLR117c were made by sub cloning the 2-Hybrid library cDNA fragment of each gene as an EcoRI fragment from pJG4-5 to a modified version of the BlueScript KS+ vector, with the polylinker removed (see Methods). The BNR1, YNL054w, and YLR117c clones were digested with Eco47III and a BglII linker inserted.
Table VII. Interactions of Fus1p (97-512aa), p1336, with Bnr1p (297-789aa), Bud2p (849-1105aa), Ynl054wp (665-916aa), Ylr117cp (274-573aa) and Ynl075wp (38-291aa) isolated from a 2-hybrid library screen with Fus1p (97-512aa) as 'bait'. Interactions were detected with the two-hybrid system (see Methods). Data (Miller units) are means ± SD of three independent experiments.
<table>
<thead>
<tr>
<th>AD fusions</th>
<th>LexA-DBD fusions</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Miller units ± SD</td>
</tr>
<tr>
<td>p1129 Bnr1p (297-789aa)</td>
<td>Fus1p (97-512aa)</td>
<td>7.93 ± 0.42</td>
</tr>
<tr>
<td>p1522 Bud2p (849-1105aa)</td>
<td>Fus1p (97-512aa)</td>
<td>12.96 ± 1.01</td>
</tr>
<tr>
<td>p1130 Ynl054wp (665-916aa)</td>
<td>Fus1p (97-512aa)</td>
<td>124.90 ± 9.37</td>
</tr>
<tr>
<td>p1521 Ylr117cp (274-573aa)</td>
<td>Fus1p (97-512aa)</td>
<td>8.30 ± 0.62</td>
</tr>
<tr>
<td>p1523 Ynl075wp (38-291aa)</td>
<td>Fus1p (97-512aa)</td>
<td>37.07 ± 2.49</td>
</tr>
<tr>
<td>p1129 Bnr1p (297-789aa)</td>
<td>pEG202 (p455)</td>
<td>0.82 ± 0.08</td>
</tr>
<tr>
<td>p1130 Ynl054wp (665-916aa)</td>
<td>pEG202 (p455)</td>
<td>0.50 ± 0.06</td>
</tr>
<tr>
<td>V39 pGAD424</td>
<td>Fus1p (97-512aa)</td>
<td>0.62 ± 0.11</td>
</tr>
</tbody>
</table>
Figure 7. Hydrophobicity plot of the predicted protein encoded by YNL054w. Hydrophobicity was calculated by the Kyte & Doolittle method (arbitrary units).

Figure 8. An alignment of the proteins encoded by S. cerevisiae YNL075w and C. elegans ZK795c.
File: YNL075w protein
Table: Kyte & Doolittle
Window: 20  Average: -0.88  Threshold Line: 0.0

Score = 393 (180.4 bits), Expect = 1.3e-91, Sum P(5) = 1.3e-91
Identities = 74/130 (56%), Positives = 92/130 (70%)

YNL075w: 81 SGIMDPRIIVTTSRDPSTRLSQFAKEIKLLFPNAVRLNREGNYVM 124
+G DP+I++TTSRDPS+RL FAKE+KL+FPNA R+NRG+Y +
ZK795c: 73 AGAQPDKIVITTSDPSRLMKMFHEMKMIFPNAQRINRGHYDV 117

YNL075w: 125 PNLVDACKKSGTDDLVVLHEHRGVPTSLTISHFPHGPTAQFSHL 168
+V A K +TDL++ E RG P + + H P GPTA FS+
ZK795c: 118 KQVVQASKAQDSTDILLIFETETRGPDMLVCLHPFGPTAFFSMA 161

YNL075w: 169 NVVMRHDIINAGNQSEVNPHLIFDNFTALGKRVVCILKHLF 210
NVVMRHDI N G SE PHLIFDN + LG R ILKHLF
ZK795c: 162 NVVMRHDIPNCGTMSSEQYPHLIFDNLSKLGHRFTILKHLF 202

Score = 93 (42.7 bits), Expect = 1.3e-91, Sum P(5) = 1.3e-91
Identities = 17/31 (54%), Positives = 22/31 (70%)

YNL075w: 214 PKKDSERVITFANRGDFISVRQHVYVRTREG 244
PK DS+R+ITF+N D+IS R HVY +G
ZK795c: 205 PKPDSDKRIITFNSEDYISFRHHSVKTENDG 235
Figure 9. An alignment of the proteins encoded by *S. cerevisiae* YLR117c and *D. melanogaster* crooked neck.
Score = 467 (215.6 bits), Expect = 8.3e-160, Sum P(8) = 8.3e-160
Identities = 82/191 (42%), Positives = 129/191 (67%)

crn: 48 ISDPAELADYQQRKRTFEDNLKRKNRMVSHWIKYAQWEQQQE 91
     ID EL +YQ+RKR +E L++NR + WI+YAQ+E +Q +
YLR117c: 37 ILDLEELREYQQRKRTTEYEGYLKRNRDLMGQWIRYQAQFEIEQHD 80

crn: 92 IQARSIWERALDNEHRVNLTLKYAEEMKNKQVNYHARNLWDR 135
     ++RARS+ERAL + + LW++Y + E+K +NHARNL +R
YLR117c: 81 MRRARSIFERALLVDSSIFLPWIRYIDAEKLNKHARMLNR 124

crn: 136 AVTIMPRVQFQWYKYTHEEMLENVAGARQVFHERMWQPEEQA 179
     A++ +PRV++ WYKY +EE L NV + ++ W ++ P A
YLR117c: 125 AISTLPVRDKLWYKYLIVEEASNNEIVRSLYTKWCSEPQVA 168

crn: 180 WQTYNFELRYKEIDRAREIYRFYVHPDVKNWFARFEESH 223
     W ++V+FE+R W + REIY ++V HP ++ W+K+ RFE H
YLR117c: 169 WNSFVDFEIRQKNWNGVREIYSVMAHPQMFTWLKWVFRENHR 212

crn: 224 GFIHGSRRVFERAVE 238
     G +R V+ A++
YLR117c: 213 GNTEFTRSVYSLAID 227

Score = 331 (152.8 bits), Expect = 8.3e-160, Sum P(8) = 8.3e-160
Identities = 57/107 (53%), Positives = 82/107 (76%)

crn: 397 ELIPHKQFTFSKLWLYAQFTEIRCKELQRARKALGLAIGCMRD 440
     ++IPKX FTFSK+WL+YA+F IR ++ +ARK LG .AIG+CP+
YLR117c: 394 DIIPHKQFTFSKLWLMYAKFLIRHDDVPKARKILGKAIGLCPKA 437

crn: 441 KLFRGYIDLEIQDLREREFRCRIEMYKFLKFCFPENGCVTWMKFAELE 484
     K F+GYI+L+L+EF+R R ++YEKF+EF P ++ W ++ELE
YLR117c: 438 KTFKGYIELEVLKKEFDRVRKIYEFKIEFQPSDLQIWSQYGELE 481

crn: 485 NLLGDDTRARAILFELAVQQ 503
     LGD DR R I+ +A+ +
YLR117c: 482 ENLDWDWVRGITYALDE 500

Score = 102 (47.1 bits), Expect = 8.3e-160, Sum P(8) = 8.3e-160
Identities = 23/70 (32%), Positives = 37/70 (52%)

crn: 293 EKKYDRAGIEDVIVSRKRYQYEQEQVAANPTNYDAWFDYLYRLIE 336
     EK++GD IE+ I KRK +YE ++ N +YD W+ YL LI
YLR117c: 288 EKQFQGIDNIEETISYRKKMETYEITLSNNAYDYDVWLWYLDSLIS 331

crn: 337 AEGDRQIREYERAISNVPPANEKN 363
     + + + + P KN
YLR117c: 332 ESFPKQIMQTTEKAIVDMPKELSKN 357
The URA3 gene was inserted as a BglII fragment to make the disruption.

The YNL075w clone possessed a convenient BamHI site into which the URA3 gene was inserted as a BglII fragment to make the disruption.

As BNR1 is a homolog of BNI1 I decided to examine whether a double mutant (bnr1Δbni1Δ) showed a lethal or more severe mating defect phenotype than a bni1Δ mutant. In order to create a bnr1Δ strain, I transformed the BNR1 gene disruption plasmid (p1527) into strain Y688, a heterozygous diploid with a disruption in one copy of BNI1, (bni1Δ::LEU2/BNI1+ α/α diploid). This allowed the phenotype of a single (bnr1Δ) and double (bnr1Δbni1Δ) mutant to be examined. The YNL054w, YNL075w and YLR117c gene disruption plasmids were transformed into SY2575, an α/α diploid, sporulated and tetrads obtained.

**Gene disruption of BNR1**

Tetrads of the BNR1 gene disruption were viable and segregated 2:2 for LEU+/URA+ (see Table VIII). From this set, bnr1ΔBNI1(Y1006, Y1007) and bnr1Δbni1Δ(Y940, Y941), MATa and α versions, respectively, disruption strains were obtained. PCR was carried out on genomic DNA extracted from these strains in order to confirm the full gene disruption of the clone of BNR1, using primers Yip91 and URA3PCRkocheck (see Methods, Figure 10).

Under light-phase microscopy the bnr1Δ strain and the bnr1Δbni1Δ strain appeared as wild-type, and at 37°C there was no apparent growth or morphological defect. Using a plate assay that scores for G1 arrest (see Methods) assays were performed on both MATa versions of the bnr1Δbni1Δ (Y940) and bnr1Δ (Y1006) gene disruption strains, and no discernable defect in α-factor sensitivity could be seen compared with wild-type W3031A (data not shown). Both Y940 and Y1006 strains were made bar1Δ by crossing to Y5
(bar1Δ), sporulating and selecting for those MATa tetrads which failed to G1 arrest with a normal halo in response to α-factor, termed Y1158 and Y1058, respectively (see Table I). Pheromone response assays were performed on both strains (see Methods), remarkably both Y1158 and Y1058 appeared to form mating projections as well as the wild-type (SY2625) bar1Δ strain (data not shown). Therefore, the bnr1Δ suppressed the depolarized and round cell shape associated with a bni1Δbar1Δ strain in the presence of α-factor. As the mating projection defect associated with a bni1Δbar1Δ cell could be suppressed by bnr1Δ, the same question was asked for the mating defect. Mating assays were performed with Y940, Y941 and Y1006, Y1007 (see Methods) with the wild-type tester strains Y63 and Y62 (see Figure 11.). The mating defect was indeed suppressed, approximately to wild-type levels based on the qualitative results. Bnr1p therefore appears to act as a negative regulator of the mating projection and fusion defect associated with a bni1Δ strain. As my disruption of BNRI was not a full-length gene disruption it is possible that a truncated portion of Bnr1p is still expressed. This protein may function in a hyperactive manner to promote pheromone induced morphogenesis, and thus restore the fusion defect in a bni1Δ background. Or, Bnr1p may act in an antagonistic manner to pheromone induced morphogenesis.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>bni1Δ</th>
<th>bnr1Δ</th>
<th>bnr1Δbni1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Markers</td>
<td>(ura/leu)</td>
<td>(ura/LEU)</td>
<td>(URA/leu)</td>
<td>(URA/LEU)</td>
</tr>
<tr>
<td>Number of spores</td>
<td>31</td>
<td>23</td>
<td>23</td>
<td>31</td>
</tr>
</tbody>
</table>

Table VIII. Tetrad data resulting from Y688 (bni1Δ::LEU2/BNI1+ α diploid) transformed with EcoRI digested p1527 (bnr1Δ::URA3) gene disruption plasmid, sporulation and subsequent tetrad dissection (see Methods).
**Figure 10.** PCR check of *bnr1Δ* gene disruption strains. 

Genomic DNA was extracted from the following strains and PCR carried out using the primers YIP9A, URA3PCRcheck and URA3upstream (see Methods).

Lanes: 1,2 = Y940; 3,4 = Y941; 5,6 = Y1006; 7,8 = Y1007;

Lanes 1,3,5 and 7 used primers YIP9A and URA3PCRkocheck to amplify a 422bp fragment, including 322bp of *BNR1* and 100bp of *URA3*. Lanes 2, 4, 6 and 8 used the same PCR conditions, but no primers were added to the reactions.

**Figure 11.** Qualitative mating assay of A) wild-type (Y3031B), B) *bni1Δ* (Y654), C) *bnr1Δ* (Y1007) and D) *bnr1Δbni1Δ* (Y941) mated to the wild type mating tester strain Y62 for four hours on YEPD+Ade before replica plating to SD medium.
Gene disruptions of YNL054w, YNL075w, and YLR117c

Gene disruptions of YNL054w and YLR117c were inviable, the tetrads segregated 2:2 for a lethal phenotype. The ynl054wΔ tetrad plates consistently demonstrated a very small colony generated from the germinated spore, for the gene disruption strain. Microscopic examination revealed a micro-colony of approximately 20 small sick looking cells, the gene disruption haploid strain could therefore divide initially, but led to a lethal phenotype after a few rounds of division. Incubating the tetrads at room temperature, rather than 30°C, did not result in any further growth. ylr117cΔ showed an even more severe lethality, no colonies were seen at all for the gene disruption tetrads.

ynl075wΔ appeared viable. Tetrads segregated 2:2 for the URA3 auxotrophic marker, however, no apparent growth or temperature sensitive phenotype was demonstrated. Neither did the ynl075wΔ demonstrate a mating defect in qualatative mating experiments, nor demonstrate a defect in G1 arrest or budding. Therefore, I have shown that at least two of the three proteins encoded by these unknown ORFs appear to be essential to the cell.

Note that neither PCR or Southern analysis on genomic DNA isolated from the ynl054wΔ, ynl075wΔ and ylr117cΔ gene disruptions have been carried out to date, therefore these mutants have not been confirmed as full gene disruptions.

Two-Hybrid experiments with Bnr1p

Following the result from another group that Bnr1p interacts with the Rho family member, Rho4, and profilin, (Imamura, H. et al., 1997) (see Discussion) I carried out the same experiment with my Bnr1p clone. The pEG202 DBD fusions of Profilin, Rho1p Q68H, Rho2p G25V, Rho3p G25V,
Rho4p G25V and Cdc42p, Cdc42p G12V were transformed into strain Y596 with DNA AD (p1129) pJG4-5::BNR1. Quick LacZ assays were performed on the transformants (see Methods). β-galactosidase activity was only shown between Bnr1p and profilin (data not shown), confirming the result shown by Imamura et al., 1997. However, as no detectable interactions were shown with Rho4, we therefore assume that our clone of BNRI does not include the Rho4p binding domain.

**Two-Hybrid experiments with Chs5p**

Like FUS1, CHS5 is important for cell fusion during mating (Favero, T., unpublished results; Santos, B. et al., 1997). The question of whether Chs5p interacts with Fus1p, actin, the MAPK pheromone response pathway or any of the other actin cytoskeleton-associated proteins was asked. Currently a model exists for CHS5 (chitin synthesis-related 5) being responsible for the transport of CHS3, and potentially other proteins, to their target destinations in the cell (Santos, B. and M. Snyder, 1996).

In order to determine whether Chs5p interacts with Fus1p or components of the pheromone response pathway, DNA activation domain fusions of the pheromone response pathway components, Ste4p, Ste5p, Ste7p, Ste11p, Ste20p, Ste12p, Fus3p, Kss1p and other actin cytoskeleton-associated components Fus2p, ACTIN, Rvs167p and Rvs161p were transformed into strain Y704 with DNA binding domain (p1608) pBTM116NLS::CHS5. Quantitative LacZ assays were performed as detailed (see Methods and Table IX). The results show a strong interaction between Chs5p and Fus3p and actin. A weaker interaction was seen with Fus1p and weaker still with Ste20p, Bem1p. This result suggests that Chs5p is forming a complex with Fus1p, Fus3p and actin.
Table IX. Interactions of Chs5p (p1608) with the MAPK pheromone response pathway components and other cytoskeletal proteins. Interactions were detected with the two-hybrid system (see Methods). Data (Miller units) are means ± SD of three independent experiments.
<table>
<thead>
<tr>
<th>Gal4p-AD fusions</th>
<th>DBD fusion</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Miller units ± SD</td>
</tr>
<tr>
<td>p1435 Fus3p</td>
<td>p1608 Chs5p</td>
<td>18.04 ± 1.60</td>
</tr>
<tr>
<td>p628 Fus1p</td>
<td>p1608 Chs5p</td>
<td>4.96 ± 0.20</td>
</tr>
<tr>
<td>p1124 Act1p</td>
<td>p1608 Chs5p</td>
<td>15.19 ± 0.62</td>
</tr>
<tr>
<td>p1438 Ste20p</td>
<td>p1608 Chs5p</td>
<td>2.44 ± 0.16</td>
</tr>
<tr>
<td>p439 Bem1p</td>
<td>p1608 Chs5p</td>
<td>1.99 ± 0.16</td>
</tr>
<tr>
<td>p974 Rvs161p</td>
<td>p1608 Chs5p</td>
<td>1.61 ± 0.21</td>
</tr>
<tr>
<td>p1432 Ste5p</td>
<td>p1608 Chs5p</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>p1423 Ste11p</td>
<td>p1608 Chs5p</td>
<td>0.13 ± 0.10</td>
</tr>
<tr>
<td>p1428 Ste7p</td>
<td>p1608 Chs5p</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>p1487 Ste12p</td>
<td>p1608 Chs5p</td>
<td>0.13 ± 0.04</td>
</tr>
<tr>
<td>p688 Bud6p</td>
<td>p1608 Chs5p</td>
<td>1.00 ± 0.16</td>
</tr>
<tr>
<td>p892 pACT2</td>
<td>p1608 Chs5p</td>
<td>0.65 ± 0.17</td>
</tr>
</tbody>
</table>
DISCUSSION

In response to a partner of the opposite mating type or exogenously added pheromone, a haploid yeast cell arrests its cell cycle and actively transcribes a set of genes required for the formation of a mating projection and subsequent cell fusion. One such gene, *FUS1*, is actively transcribed over 100-fold in response to pheromone. The protein product, Fus1p, appears to be a key molecule during the remodelling and subsequent fusion of the cell walls and plasma membranes of the two mating partners (the prezygote) following conjugation (McCaffrey, M. et al., 1987; Trueheart, J. et al., 1987). Fus1p is of particular interest as it possesses an SH3 domain at its COOH-terminus, lying on the cytoplasmic side of the plasma membrane. SH3 domains have been implicated in protein-protein interactions between cytoskeletal and signal transduction molecules.

Cell fusion in *S. cerevisiae* is a relatively unstudied event of the yeast mating response. In this study I chose a two-hybrid approach to identify what other molecules may interact with Fus1p, in order to further our understanding of the actual role Fus1p plays during the mating and cell fusion event. Based upon the protein-protein interactions between gene products involved in actin mediated reorganization, including Rvs167p, Chs5p and Fus2p, and components of the MAPK pheromone response cascade, Fus3p, Ste5p, Ste7p and Ste11p, I have defined a putative "fusion complex" which links the cytoskeleton and the activated signal transduction machinery to the site of highest pheromone concentration (ie. the tip of the mating projection).
Multicopy suppressor search

A search for multi-copy suppressors of the fusion defect associated with \textit{afus1Afus2} strain, isolated \textit{FUS1, FUS2, SST2}, and the \textit{a-factor} structural genes \textit{MFA1} and \textit{MFA2}. I expected to identify \textit{SST2, FUS1} and \textit{FUS2} as multicopy suppressors in this background. The suppression of the fusion defect associated with a \textit{fus1Afus2} \textit{x fus1Afus2} mating by overexpression of either \textit{FUS1} or \textit{FUS2} on a multicopy plasmid has been previously demonstrated (Trueheart, J. \textit{et al.}, 1987), however, this result demonstrates the ability of my synthetic-sterile approach to isolate mating, and fusion specific genes as suppressors. I also show that overexpression of the pheromone precursor genes, in this case for \textit{a-factor}, can alleviate the mating defect of a \textit{fus1Afus2} background. This is most likely due to the higher copy number of \textit{MFA1} and \textit{MFA2} resulting in a higher local concentration of secreted \textit{a-factor} at the junction between the mating cells, and therefore increases the efficiency of cell fusion. This result is in agreement with the recent result that the fusion defect associated with two fusion mutants, \textit{fus5} and \textit{fus8}, could be partially rescued by multicopy \textit{MFA1} (Brizzio, V. \textit{et al.}, 1996). \textit{FUS5} and \textit{FUS8} were allelic to \textit{AXL1} and \textit{RAM1/DPR1}, respectively, two genes known to be involved in the \textit{a-factor} biogenesis pathway. Also, recently a mutant allele of the \textit{a-factor} ABC transporter \textit{STE6, ste6-cefl}, has been reported that shows a strong \textit{a-specific} unilateral cell fusion defect (Elia, L. and L. Marsh, 1996). In the light of these results, it appears that a high threshold of pheromone (\textit{a-factor} and \textit{\alpha-factor}) is required to signal the partner cell to undergo cell fusion following prezygote formation.
Two-Hybrid interactions between Fus1p, Rvs167p and the MAPK pheromone response cascade

This study has demonstrated that Fus1p interacts in vivo, in the two-hybrid system, with the cortical actin endocytotic vesicle associated protein Rvs167p, Fus1p itself, the fusion related Fus2p, the MAPKs Fus3p and Kss1p, the MAPKKK Ste11p, the MAPK scaffold protein Ste5p, and more weakly with the MAPKK Ste7p (see Table V, Table VI).

Rvs167p, a putative link to actin-based endocytotic vesicles

Rvs167p contains one conserved SH3 domain at its COOH-terminus, and has been shown to interact with actin in the two-hybrid system (Amberg, D. C. et al., 1995). Previously rvs167Δ mutants have been shown to possess an abnormal actin distribution, particularly under high salt (stress) conditions (Bauer, F. et al., 1993). Although the gene RVS167 and its homologue RVS161 play a role in response to nutrient stress in unfavourable conditions, they show pleiotropic mutant phenotypes, including endocytosis and budding defects (Crouzet, M. et al., 1991; Bauer, F. et al., 1993; Durrens, P. et al., 1995; Sivadon, P. et al., 1995). This points towards a more general role for both homologs in the control of actin-based endocytosis and actin organization.

The presence of a GPA rich region and the SH3 domain in Rvs167p implicates that, more specifically, it functions in cortical actin cytoskeleton organization (Drubin, D. G. et al., 1990). Recently Rvs167p has been shown to bind the S. cerevisiae actin-binding protein Abp1 via its SH3 binding domain (Lila, T. and D. G. Drubin, 1997). Abp1 also appears to play a role in the cortical actin cytoskeleton, it too possesses an SH3 domain, and appears to mediate the
normal association of the adenylyl cyclase-associated protein Srv2p with the cortical actin cytoskeleton.

Both Rvs161p and Rvs167p show similarity to amphiphysin, a neuronal protein which has been associated with synaptic vesicles and has been identified as the autoantigen of Stiff-Man Syndrome, a condition associated with breast cancer. Therefore Rvs161p and/or Rvs167p may associate with endocytotic vesicles and be required for vesicle fusion during cell membrane fusion (Dorer, R. et al., 1997). The process of cell fusion itself requires degradation of the cell walls of the two mating partners and formation of the pre-zygote. This is a highly critical process for the cell as the dissolution of the cell wall leaves it vulnerable to lysis and to its environment. In view of this, the cell fusion event must be very well orchestrated, and monitored.

Rvs167p, possibly in association with Rvs161p, may therefore form a link between the point of plasma membrane fusion at the projection tip, via membrane bound Fus1p, to the cortical actin cytoskeleton. Further genetic evidence for this model is provided from this study by the slight mating defect shown with a fus1Δrvs167Δ mutant (see Figure 5). The combination of a mutation in both RVS161 and RVS167 results in a slight mating defect, whereas a mutation in either gene alone demonstrated no such defect.

Two-Hybrid interactions between Fus1p and the MAPK pheromone response pathway

The MAP kinase Fus3p demonstrated a strong interaction with Fus1p. As Fus3p is required for cell fusion, does this mean that it has a function in the process of cell fusion independent from Fus1p; or that now a direct interaction between the two proteins is seen, that Fus3p may possibly
phosphorylate and so activate Fus1p? Experiments to study the phosphorylated state of Fus1p in response to pheromone, and in a fus3Δ background need to be carried out to investigate this.

Fus1p interacted strongly with Kss1p, the functionally related MAP kinase to Fus3p. KSS1 is required for signal transduction, but not for cell fusion. However, as Fus1p, and therefore the "fusion complex", localizes to the point of the mating projection where the strongest pheromone signal is detected, it is feasible that Fus1p may interact with Kss1p.

Ste5p and Ste11p demonstrated an interaction with Fus1p, confirming that Fus1p interacts with many of the components of the MAPK module. The interaction between Ste11p and Fus1p required a catalytically active version of Ste11p; β-galactosidase activity was lost when a catalytically inactive version, Ste11 K444R, was tested (see Table VI). The interactions between Fus1p and Ste5p, and Fus1p and Ste11p were lost when the two-hybrid interactions were examined in a ste7Δ or a ste11Δ mutant background. These results indicate that Ste5p, Ste11p and Ste7p have to remain in a complex, and at least Ste11p has to be catalytically active, in order to interact with Fus1p. Loss of either Ste7p or Ste11p will therefore prevent the MAPK components from forming a complex, which will result in the cell being unable to respond to a pheromone gradient and transcribe mating specific genes.

An important factor to consider at this stage is the degree of sensitivity shown with the two-hybrid system. Table V, VI, VII and VIII show the quantitative level of β-galactosidase activity expressed in Miller units, for two-hybrid interactions between Fus1p and selected proteins. Clearly a high Miller unit signifies a positive two-hybrid interaction, however a high value does not signify a 'stronger' interaction than a lower value. Factors including the level of expression of the specific protein in the cell; localization of the protein in the nucleus of the cell; and the possibility that a positive two-
hybrid interaction may be favourable in the nucleus, but unfavourable in the cytoplasm, due to ionic conditions for example, may all result in differences in the level of sensitivity shown by this assay, and potentially lead to false-positives or false-negatives. In Table VI it is clear that a positive two-hybrid interaction occurs between Fus1p and the proteins Fus3p, Kss1p, Ste5p, Ste11p, and Ste7p as well as between Fus1p itself, judged by the high Miller unit values. The negative control, vector alone (pACT2), showed a value of less than one unit, however values up to three units were measured, with Ste7p for example. In order to be consistent, I decided on a 'cut-off' point of three Miller units for positive interactions. This was important to consider as I wanted to be certain that the interactions that I reported as positive were unquestionably so: Reproducibility is a concern, therefore three independent qualitative measurements were made for each protein tested.

In this study it was not possible to specify which domains of Fus1p were required for the interactions observed. Truncated versions of Fus1p: lacking the C-terminal SH3 domain (97-473aa); lacking the N-terminus (312-512aa); and including only the SH3 domain (410-512aa), all demonstrated no interaction with the proteins that did interact with Fus1p (97-512aa). Possibly some, if not all, of these truncations are incorrectly folded or functionally inactive and so are incapable of demonstrating a two-hybrid interaction. The role of the SH3 domain of Fus1p therefore needs to be further examined. This would be most elegantly carried out by making point mutations in the conserved region of the SH3 binding domain, that renders the domain non-functional. This should demonstrate whether the SH3 domain is required for Fus1p to associate with any of the aforementioned proteins.
Two-Hybrid interactions between Fus2p and the MAPK pheromone response pathway

Fus2p also appears to form part of this putative "fusion complex" with Fus1p. Two-hybrid experiments demonstrate that Fus2p interacts strongly with Fus1p, Rvs161p, and Rvs167p, (Evangelista, M., personal communication). Fus2p also interacts with the MAPKKK Ste11p, the MAPKK Ste7p and the MAPK Kss1p, but no interaction was demonstrated with Fus3p or Ste5p. These results may explain the ability of FUS1 or FUS2 to suppress a mutation in the other gene. As Fus1p and Fus2p form a complex together with specific proteins to promote cell fusion, then the loss of either protein is compensated for by the presence of the other; based upon their similar 'profiles' in two-hybrid protein-protein interactions.

Two-Hybrid library screen with Fus1p

Screening a yeast genomic library with Fus1p (97-512aa) resulted in the isolation of five positively interacting proteins, encoded by BNR1, BUD2, YNL054w, YNL075w and YLR117c.

BNR1

BNR1 (Yip9/YIL159w) is a homolog of BNI1, the yeast formin (Evangelista, M. et al., 1997) and shares the formin homology domains FH1 and FH2 which are found in related genes in other organisms, including cappuccino (Emmons, S. et al., 1995) in Drosophila melanogaster, fus1 in Schizosaccharomyces pombe (Petersen, J. et al., 1995) and FigA in Aspergillus nidulans (Marhoul and Adams, 1995). All of these related genes have been shown to be involved in cytokinesis, cell polarity establishment, or cell
morphology. In this study a two-hybrid interaction was shown between Fus1p and the region of \textit{BNR1} including the formin homology 1 (FH1) domain. This domain consists of proline-rich sequences which have been previously shown to bind to an SH3 domain (Ren, R. et al., 1993) and profilin (Tanaka, M. and H. Shibata, 1985). By making a gene disruption of \textit{BNR1}, I discovered that the gene is not essential, and did not appear to show any growth, mating (fusion), or budding defect in our strain background, W303, or in two other strain backgrounds (data not shown). An interesting result was seen when testing the \textit{bni1\Delta bnr1\Delta} double mutant strain in a mating assay. The strong mating defect of a \textit{bni1\Delta} appeared to be suppressed, almost to wild-type levels, by making the double mutant (see Figure 11). Examining the ability of the \textit{bni1\Delta bnr1\Delta} strain to form mating projections in response to exogenous pheromone revealed that the cells appeared to shmoo as wild-type, as opposed to \textit{bni1\Delta} mutants which fail to undergo polarized morphogenesis (Evangelista, M. et al., 1997). This demonstrates that Bnr1p participates, in some manner, with Bni1p and the cell polarization machinery. I also tested for two-hybrid interactions between Bnr1p; Bni1p and Axl1p. Bnr1p showed a strong interaction with Axl1p, the yeast gene product that shares homology with the insulin-degrading enzyme family of endoproteases. Axl1p has two distinct functions, it is responsible for axial bud site selection (Fujita et al., 1994) and for a-factor propheromone processing (Adames et al., 1995). This result, in combination with the interaction shown between Fus1p and Bud2p, suggests that there may be a link between Fus1p, the cell fusion machinery, and proteins of the bud-site selection machinery.

Recently Bnr1p was shown to interact in both yeast two-hybrid studies and biochemically with the actin-binding protein, profilin, through its FH1 domain (Imamura, H. et al., 1997). In the same study it was demonstrated that \textit{bni1\Delta bnr1\Delta} mutant cells arrested with a severe temperature-sensitive
growth phenotype, deficient in bud emergence, showing a random 
distribution of cortical actin patches and often became multinucleated. This 
phenotype was not seen in my gene disruption. This may be due to the effect 
of different strain backgrounds used, or that the gene disruption that I made 
was not a full disruption. My gene disruption of BNR1 was not made using a 
full length clone, but using the clone that was isolated from the two-hybrid 
search (amino acids 297-789). This may mean that my gene disruption of 
BNR1 was still partially functional, although my suppression result argues 
that at least one function was disrupted in the protein.

I examined if the clone of BNR1 from my two-hybrid search could 
interact with Bni1p, profilin, Rho1p, Rho2p, Rho3p, Rho4p and Cdc42p in 
two-hybrid experiments. Bnr1p (297-789aa) showed a strong interaction with 
profilin, meaning that my construct did possess enough of the FH1 domain to 
allow for an interaction to occur. My BNR1 construct encodes 21 amino acids 
of the 82 amino acid long FH1 domain (see Figure 6.), this includes a proline 
rich sequence. This proline rich sequence may therefore be essential for the 
interaction with profilin. No two-hybrid interaction was shown with Rho4p. 
As my BNR1 construct is missing the first 297 amino acids of the amino-
terminus, this region may contain the Rho4p-binding domain, based upon 
the fact that the homologous Bni1p possesses a Rho1p-binding domain at its 
amino-terminus (90-343aa).

BUD2

BUD2 was isolated once from the library screen. It encodes a GTPase-
activating protein for Rsr1p/Bud1p, the Ras related GTP-binding protein 
which is involved in bud-site selection (Bender, A. and J. Pringle, 1989; Chant, 
J. and I. Herskowitz, 1991). Bud2p, together with Rsr1p and Bud5p, are 
required for correct bud-site selection, regardless of cell type; haploid and
diploid bud2 mutants show a random budding phenotype (Park, H-O et al., 1993). Recently, however, bipolar budding-specific alleles of BUD2 and BUD5 have been identified which remain capable of interacting with the Bud3p/Bud4p-containing axial signal (Zahner, J. et al., 1996). This suggests that both Bud2p and Bud5p possess domains that are specific for interaction with both axial-specific and bipolar-specific positional signals.

The fact that a protein required for bud-site selection interacts with Fus1p suggests that there is a link between the budding pathway and the cell fusion pathway. Further evidence for a tentative link between the mating and budding pathway has been demonstrated by a mating deficiency in an rsr1Δ background (Marsh, L., personal communication). In view of this result I tested for two-hybrid interaction with AD Fus1p and AD Fus2p against DBD versions of Rsr1p, Rsr1p G12V, Rsr1pK16N, Bud2p, Bud3p, Bud5p, Bud10p, and Axl1p. However, no interactions were detected except between Fus1p and Bud2p, based upon β-galactosidase activity. Although there is no direct interaction between Fus1p and the other components of the budding pathway, there is now evidence that components of the budding pathway play a role in the cell fusion/mating pathway. Therefore, two morphogenetically distinct pathways that result in the development of cellular polarity in yeast, may in fact require common proteins.

**YNL054w, YLR117c and YNL075w**

YNL054w, YLR117c and YNL075w are three previously unidentified ORFs encoding putative protein products that were isolated from the library screen with Fus1p.

Ynl054wp showed no homology to any current proteins in the databases when examined via BLASTP searches. One indication of its
function is that it possesses a putative C-terminal transmembrane domain, owing to a stretch of hydrophobic residues. Therefore Ynl054wp may be localized to the plasma membrane in order to carry out its function. Making a *ynl054wΔ* gene disruption version, demonstrated that it is a putative essential gene. The cells from each gene disruption in a tetrad appeared capable of undergoing a few rounds of division before senescence, which may indicate that the protein has a structural role in the cell wall or plasma membrane.

Ylr117cp showed similarity to the *D. melanogaster* protein encoded by *crooked neck (crn)*, when compared with the current protein databases via BLASTP searches (Zhang, K. *et al.*, 1991)(see Figure 9). The similarity is due to an amino-acid tandemly repeated sequence that is found in 16 copies in the protein encoded by *crn*. This amino acid repeat is similar to the tetratrico peptide repeat (TPR) motif that was found in a family of fungal genes (Boguski *et al.*, 1990; Hirano *et al.*, 1990; Sikorski *et al.*, 1990), including four cell cycle related genes, *CDC16, CDC23, nuc2*, and *BimA*. However, the apparent functions of TPR-containing proteins are quite diverse, including protein transport, RNA splicing, and regulation of gene expression.

Loss of maternal expression of *crn* results in the absence of the female germ line, which may be due to defects in cell proliferation. Loss of zygotic expression of *crn* leads to defects in the proliferation of brain neuroblasts and results in the absence of specific neuronal lineages in the central and peripheral nervous systems (CNS and PNS, respectively) of the embryo (Zhang, K. *et al.*, 1991). Making a *ylr117cΔ* gene disruption version demonstrated that *YLR117c* is another putative essential gene. The cells from each tetrad of a gene disruption did not grow at all, demonstrating the lethality of a loss in function.

Ynl075wp showed significant sequence similarity to a putative protein encoded by an ORF present in *Caenorhabditis elegans*(see Figure 8).
Searching the current databases with the Ynl075wp sequence revealed that this sequence appears to be conserved in mammals, including *H. sapiens*. However, no matches were made to any known proteins.

Making a *ynl075wΔ* gene disruption did not result in lethality. The gene disruption did not appear to affect growth, mating, growth arrest in response to pheromone or budding. Further experiments are required in order to make a full gene disruption of *ynl075wΔ* and attempt to determine its function.

Therefore in a two-hybrid library search with Fus1p I have found three novel putative proteins encoded by the ORFs *YLR117c, YNL075w* and *YNL054w*. One shows significant similarity to a *D. melanogaster* protein that functions in cell proliferation, another appears to be strongly conserved in higher eukaryotes, including *C. elegans*, *M. musculus*, and *H. sapiens*, and another possesses a potential membrane spanning domain. Disruptions of the genes encoding by two of these proteins resulted in lethality, demonstrating that they are putative essential genes. Further work is therefore required to ascribe a function to the genes, in particular *YNL075w* and to determine what role they might play in the mating and/or cell fusion event.

**Two-Hybrid interactions between Chs5p, Fus1p, actin and the MAPK pheromone response pathway**

Recently, *CHS5* was identified as a gene involved in both chitin synthesis and mating (Santos, B. *et al.*, 1997). A centromeric copy of *FUS1* or *FUS2* was found to improve the mating efficiency of *chs5Δ* cells, thus implicating an association between the fusion machinery and Chs5p. There is evidence for selectivity, as the MAPK *FUS3* which is required for cell fusion,
projection formation and G1 phase arrest, on the other hand, did not show any suppression of the chs5Δ defect. In this study, Chs5p was demonstrated to interact with Fus1p, Fus3p and Actin in two-hybrid experiments. This result suggests that Chs5p, Fus1p, Fus3p and actin are closely associated with one another in the cell. This provides further evidence to support the previous model that Chs5p delivers not only Chs3p, but other proteins required for cell wall remodelling or cell fusion (Santos, B. and M. Snyder, 1997).

The final model

I suggest a model that Chs5p is associated with transport vesicles that deliver Fus1p and Fus3p to the site of cell fusion, i.e. the tip of the mating projection, potentially in an-actin dependent manner. Once at the site of cell fusion, Fus1p and Fus3p form part of a "fusion complex" with other cell fusion and cytoskeletal associated proteins, including the MAPK pheromone response pathway components Ste11p, Ste7p, Ste5p and Kss1p, Fus2p, and Rvs167p and Rvs161p, both homologues of a mammalian synaptic vesicle associated protein functioning at the level of neurotransmitters.

The interaction between Fus1p and the Bni1p homologue, Bnr1p, suggests a further link between the fusion machinery and components that regulate reorganization of the actin cytoskeleton. Bnr1p has been demonstrated to interact with the Rho family member, Rho4p, and profilin, an actin-binding protein (Imamura, H. et al., 1997).

Exactly how the components of this complex control the process of cell fusion remains a fascinating question. Certainly, proteins are required in order to remodel the cell wall in preparation for the cell fusion event, which is expected to occur in a cytoskeletally regulated manner. Potentially the
protein products of the three genes YNL054w, YNL075w and YLR117c, may be involved in this remodelling process.

This study provides evidence that the process of cell fusion during mating in Saccharomyces cerevisiae requires the formation of a novel "fusion-complex", which provides a link between the cytoskeletal and fusion machinery to the site of highest pheromone concentration (see Figure 12). Molecules required for this complex may be targeted to the membrane in an actin dependent manner via endocytotic synaptic vesicles in a manner analogous to the transmission of neural peptides in the nerve synapse.
Figure 12. A model to show the putative "fusion-complex" formed between components of the MAPK pheromone response cascade, Fus1p, Fus2p, Rvs161p, Rvs167p and Chs5p, based upon two-hybrid analysis of protein-protein interactions. A possible link to the actin cytoskeleton is shown (shaded) through Rvs167p.
REFERENCES


Cherry, J. M., C. Adler, C. Ball, S. Dwight, S. Chervitz, Y. Jia, G. Juvik, T. Roe, 
S. Weng and D. Botstein. 1995. "Saccharomyces Genome Database" 
http://genome-www.stanford.edu/Saccharomyces/

Cid, V. J., A. Durán, F. Del Rey, M. P. Snyder, C. Nombela and M. Sánchez. 
1995. Molecular basis of cell integrity and morphogenesis in 

G1 arrest response in cells expressing an inappropriate pheromone 

viability upon nutrient starvation: characterization and cloning of the 

Crouzet, M., A. Breton, M. Bonneu, L. Desfarges, P. Durrens, P., Sivadon and 

De Camilli, P., A. Thomas, R. Cofiell, F. Folli, B. Lichte, G. Piccolo, H-M. 
Meinck, M. Austino, GH. Fassetta, G. Bottazzo, D. Bates, N. Cartlidge, 
M. Solimena and M. Kilimann. 1993. The synaptic vesicle-associated 
protein amphiphysin is the 128-kD autoantigen of stiff-man syndrome 

Inhibition of G-protein signalling by a dominant gain of function 
mutations in Sst2p, a pheromone desensitization factor in 

execute a default pathway to select a mate in the absence of pheromone 


*Cell* 60: 319-328.


