FH3, A Domain Found in Formins, Targets the Fission Yeast Formin Fus1 to the Projection Tip During Conjugation

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Abstract. Formins are involved in diverse aspects of morphogenesis, and share two regions of homology: FH1 and FH2. We describe a new formin homology region, FH3. FH3 is an amino-terminal domain that differs from the Rho binding site identified in Bni1p and p140mDia. The *Schizosaccharomyces pombe* formin Fus1 is required for conjugation, and is localized to the projection tip in cells of mating pairs. We replaced genomic *fus1*⁺ with green fluorescent protein (GFP)-tagged versions that lacked either the FH1, FH2, or FH3 domain. Deletion of any FH domain essentially abolished mating. FH3, but neither FH1 nor FH2, was required for Fus1 localization. An FH3 domain–GFP fusion protein localized to the projection tips of mating pairs. Thus, the FH3 domain alone can direct protein localization. The FH3 domains of both Fus1 and the *S. pombe* cytokinesis formin Cdc12 were able to localize GFP to the spindle pole body in half of the late G2 cells in a vegetatively growing population. Expression of both FH3-GFP fusions also affected cytokinesis. Overexpression of the spindle pole body component Sad1 altered the distribution of both Sad1 and the FH3-GFP domain. Together these data suggest that proteins at multiple sites can interact with FH3 domains.

Execution of the correct morphogenic program is essential for the growth fidelity of eukaryotes, be it during complex developmental processes such as limb formation, or in linear cell extension in fungi. Polarization of individual cells in response to diverse signals, e.g. internal programs, external factors, or cell-cell contact, can simply be defined as the generation of asymmetric distribution of specific molecules or factors that direct global alterations in the cytoskeleton. In simple eukaryotes such as the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe,* the actin cytoskeleton plays a key role in establishing and maintaining polarized growth, and in executing cell division (reviewed in Bretscher et al., 1994; Robinow and Hyams, 1989). Structural studies of yeast actin show that there are two types of actin filaments: cytoplasmic cables and cortical dots (Kilmartin and Adams, 1984; Marks and Hyams, 1985). Cortical dots cluster at the growing tip and the cytokinetic ring, while cables extend from the tip towards the main body of the cell. In *Saccharomyces cerevisiae*, it has been demonstrated that F-actin cortical dots are motile, responding rapidly to external stimuli (Waddle et al., 1996; Doyle and Botstein, 1996).

Several recent observations suggest that the members of the formin protein family are important for actin-related processes during polarization in diverse systems. In vertebrates, the founder member, formin, plays a key role in limb development, and p140mDia is involved in regulating actin polymerization. The *Drosophila* formins *diaphanous* and *cappuccino* execute roles in cytokinesis and polarity establishment. In fungi, formins play key roles in polarized growth and cytokinesis. Budding yeast Bni1p and Bnr1p are required for bud site selection and cytokinesis, while the *S. pombe* formin Cdc12 and the *Aspergillus nidulans* FIGA/SEPA are both required for cytokinesis (Marhoul and Adams, 1995; Woychik et al., 1990; Jackson-Grusby et al., 1992; Castrillon and Wasserman, 1994; Emmons et al., 1995; Evangelista et al., 1997; Imamura et al., 1997; Chang et al., 1997; Harris et al., 1997; Watanabe et al., 1997). Two regions of sequence homology—formin homology regions 1 and 2 (FH1 and FH2, respectively)—are found in all formins. FH1 is a proline-rich sequence that has been postulated to interact with profilin (Evangelista et al., 1997; Jansen et al., 1996; Chang et al., 1997; Imamura et al., 1997), and FH2 is defined by a consensus sequence (Emmons et al., 1995).

Bni1p interacts with a number of molecules that are important for polarized growth in budding yeast (reviewed in Chant, 1996; Roemer et al., 1996). Bni1p has been shown to interact directly with Rho1p, Cdc42p, actin, and the two

actin-binding proteins profilin and Bud6p (Kohno et al., 1996; Evangelista et al., 1997). Cdc42p is a Rho-family GTPase that is required for establishing cell polarity during the mitotic cell cycle, and for mating (Adams et al., 1990; Simon et al., 1995). Cdc42p localizes to the projection tip during mating in an actin-independent manner (Ayscough et al., 1997). Therefore, the interactions between Cdc42p and Bni1p and between Bni1p and actin suggest that this *S. cerevisiae* formin homolog serves as a link between the actin cytoskeleton and actin-independent polarization, and thus probably plays a key role in directing markers to the cell tip. Recent studies provide further evidence for such a role for formins, as the other *S. cerevisiae* formin, Bnr1p, binds to the GTPase Rho4p and the actin-binding protein profilin (Imamura et al., 1997).

The *S. pombe* formin homolog Fus1 is required for cell fusion during mating (Bresch et al., 1968; Petersen et al., 1995). Upon nitrogen starvation, diffusible mating pheromones induce polarized cell growth in cells of the opposite mating types, *P* and *M*, towards one another. Upon contact and agglutination, the cells grow towards one another, and localized cell wall degradation between the partner cells at the projection tips results in cell fusion, enabling karyogamy. After karyogamy, the resulting diploid zygote enters meiosis and sporulates (reviewed in Nielsen and Davey, 1995). Conjugation is blocked after agglutination and formation of the projection tip in the *fus1.B20* mutant, and the cell walls separating the mating partners are not degraded (Petersen et al., 1995). Thus, *fus1* mutants are blocked at the prezygote stage with a characteristic fus⁻ phenotype (two touching cells attempt to mate, but the cell wall between them remains intact).

The ability to study a formin homolog that is required for an inducible process, and hence is nonessential for normal mitotic growth, has enabled us to ask a number of key questions about the domain structure of formins. We have documented interactions of Fus1 with the actin cytoskeleton, and propose potential functions for different portions of the molecule. We have identified a new formin homology domain, which we call the FH3 domain. We use fusions of the FH3 domains of both Fus1 and Cdc12 to green fluorescent protein (GFP) to confirm the prediction, arising from deletion analyses, that the FH3 domain targets formins to their site of action.

Table I. S. pombe Strains Used in This Study

Strain	Genotype	Source or Reference
EG 282	*h⁹⁰*	Nielsen and Egel, 1990
EG 325	*h⁹⁰ura4-D18*	Nielsen et al., 1992
EG 439	*h⁹⁰ fus1::ura4⁺ ura4-D18*	Petersen et al., 1995
EG 543	*h⁹⁰mat2,3::LEU2 mam2*	This study*
EG 544	*h⁻ mat2,3::LEU2*	Egel et al., 1994
EG 545	*h⁺ mat2,3::LEU2*	Nielsen et al., 1992
EG 640	*h⁹⁰ ura4-D18 leu1*	Kjærulff et al., 1994
EG 712	*h⁹⁰ fus1-B20*	Petersen et al., 1995
EG 919	*h⁻ cdc3-124*	This study‡
EG 920	*h⁺ mat2,3::LEU2 cdc3-124*	This study
EG 938	*h⁹⁰ ura4-D18fus1-GFP ura4⁺*	This study
EG 940	*h⁺ cdc3-124 ura4-D18 fus1-GFP ura4⁺*	This study
EG 999	*h⁹⁰ fus1::LEU2 ura4-D18 leu1*	This study

*The *mam2* allele was described by R. Egel (1973*b*). ‡The *cdc3-124* allele was described by Balasubramanian et al. (1994).

Materials and Methods

Strains, Media, and Genetic Methods

The *S. pombe* strains used are listed in Table I. Cells were grown in minimal sporulation media liquid (MSL), MSL-N, MSA (minimal sporulating liquid/agar), or AA dropout media (Egel et al., 1994; Rose et al., 1990). Standard classical and molecular genetic techniques for *S. pombe* were used as described previously (Gutz et al., 1974; Moreno et al., 1991). Mating assays were performed, and mating efficiencies were calculated according to Petersen et al. (1995; *n* = 500). To determine the effect of the termperature-sensitive *cdc3.124* mutation upon Fus1 localization, cells were induced to mate at 32°C as described in Petersen et al. (1998). 32°C is restrictive for mitotic growth and conjugation of the *cdc3.124* mutant.

Molecular Manipulations

Standard procedures for bacterial and DNA manipulation were carried out according to Sambrook et al. (1989). *E. coli* DH5 (Hanahan, 1985) was used for propagation of plasmids. PCR amplification was carried out in 50-μl reaction mixtures (Kocher et al., 1989), and the PCR fragments were sequenced. The sequences of the primers used are listed in Table II. The carboxy-terminal GFP tagged Fus1: the 3′-end of *fus1* was generated by PCR using two *fus1*-specific primers (JPP1 and JPP20). This PCR product contained a 3′ BamHI site in frame with the BamHI of GFP in pDdgfp (gift from J. Haselhof). The *fus1* PCR product and a BamHI-XhoI fragment encoding GFP from pDdgfp were cloned into pDW227 (Weilguny et al., 1991), containing *ura4⁺* as a marker generating pJP67. Replacement of the *fus1* 3′-end in the genome with the GFP fusion was achieved by integrating pJP67 in EG325 to generate strain EG937. pJP67 was linearized with XbaI (Petersen et al., 1995) before transformation, and ura⁺ prototrophs were selected.

The three constructs containing deletion of the FH1, FH2, or FH3 domain in the Fus1-GFP fusion protein (see Fig. 6), were generated by combining upstream and downstream fragments (either genomic or PCR generated), which were finally fused to GFP by cloning into pJP67 to generate plasmids pJP94 (FH1), pJP93 (FH2 + coiled coil), and pJP99 (FH3). Deletion of FH1 in pJP94 (aa residues 808–817) was obtained by sequence overlapping extension PCR (Ho et al., 1989) using the two primers JPP24 and JPP25, followed by digestion with PstI. Deletion of FH2 + coiled coil in pJP93 (aa residues 1014–1274) was done by combining an upstream PCR fragment (primer JPP34) and a downstream genomic fragment starting at the HindIII site at position 3820 bp (Petersen et al., 1995). Deletion of FH3 in pJP99 (aa residues 192–411) was done by combining an upstream PCR fragment (primer JPP35) and a downstream genomic fragment starting at the XhoI site at position 1233 bp (Petersen et al., 1995). Replacement of *fus1⁺* in the genome with the various deletions mutants was achieved in EG325 by homologous recombination using the *ura4⁺* gene as a selective marker. Before transformation, the plasmids were linearized at a restriction site upstream of the various deletions, which results in only one copy of the gene under control of the *fus1⁺* promoter, since all plasmids lack the *fus1⁺* promoter and the first part of the gene. For deletions of FH1 and FH2 + coiled coil, the plasmids were linearized with EcoRI (Petersen et al., 1995), while for deletion of FH3 the plasmid was linearized at the NdeI site, which lies upstream of FH3. All integrations were confirmed by PCR and enzyme digestion of the PCR fragment.

Table II. Primers Used in This Study

Primer	Sequence (5′-3′)
JPP1	CGCGAATTCATTGATGGGAATCCAT
JPP20	CGCGGATCCCTTAAGTTCATTGTTATT
JPP24	CTCCTGCAGCATTACCACCTCCTGCAGCTCCTTTA
JPP25	TAAAGGAGCTGCAGGAGGTGGTAATGCTGCAGGAG
JPP34	CGCAAGCTTTTTCACTTACTTCCATTA
JPP35	GCGCTCGAGCTTCAACGGTGTACAGCT
JPP36	CGCGGATCCATGATGACGGCTAGTTTTAAA
JPP37	GCGCATATGGAATCTTTGGCGGTT
JPP38	CCGGGATCCGAGTAAAGCAGTTTT
JPP48	GCGCATATGATCACACTTTCTTCTTTATTATC
JPP49	CCGGGATCCAAGGTCAAATCCTTGCTC

fus1+-specific primers (JPP37 and JPP38) and PCR were used to place the Fus1 FH3 region (residue 196–382 aa) under the control of the *nmt1+* promoter in pREP2 (Maundrell, 1993) resulting in pJP102. The PCR-generated FH3 region was fused in frame to GFP by inserting the BamHI-XhoI fragment from pDdgfp into pJP102, generating pJP103. The Cdc12 FH3 region (aa residues 316–527) was cloned by PCR with *cdc12+*-specific primers (JPP48 and JPP49), and was fused in frame to the carboxy terminus of GFP in pREP42GFP (Griffiths et al., 1995; pJP106).

To do a complete deletion of *fus1* from the genome, the *Saccharomyces cerevisiae LEU2* gene from pSL19 (kindly provided by Antony Carr, Medical Research Council Cell Mutation Unit, Sussex University, United Kingdom) was cloned into Sph1-digested pDW375 (Petersen et al., 1995), creating pJP98. The HindIII fragment of pDW234 (Petersen et al., 1995) was cloned into HindIII-digested pJP98 (pJP101). pJP101 was used to delete the *fus1+* gene by homologous recombination in EG640 (Kjærulff et al., 1994) using LEU2 as a selective marker (EG999). The integration was confirmed by PCR.

Generation of Anti-fus1 Antibodies

The amino-terminal portion of the *fus1+* gene (1–1712 bp; Petersen et al., 1995) was cloned by PCR into pGEX2T (Pharmacia Biotech, Inc., Piscataway, NJ) by using a *fus1*-specific primer (JPP36) that generated a BamHI site at the initiating ATG codon. The glutathione S transferase (GST)-Fus1 fusion protein was incorporated into inclusion bodies upon induction in *E. coli BL21,* enabling the GST-Fus1 fusion protein to be purified as described in Studier et al. (1990) and used for production of polyclonal antibodies. Antibodies were affinity-purified from sera of two rabbits—1446 and 1447—using nitrocellulose-immobilized GST-Fus1 (Harlow and Lane, 1988). Both sets of antibodies stained immunoblots identically. Antibodies from 1447 were used for immunofluorescence microscopy.

Immunolocalization

For fluorescence microscopy of conjugating cells, cells were grown in MSL to a density of 5×10^6, washed in MSL -N, and starved in MSL -N for 5 h. Actin was localized by fluorescence microscopy using rhodamine-conjugated phalloidin (Marks and Hyams, 1985) after fixation in 3% formaldehyde (Hagan and Hyams, 1988) in PM buffer (Marks and Hyams, 1985). Affinity-purified anti-Cdc3 antibodies (Balasubramanian et al., 1994) were used to visualize Cdc3 by combined formaldehyde and glutaraldehyde fixation (Hagan and Hyams, 1988) in PM buffer (Marks and Hyams, 1985). Fus1, Sad1, and γ tubulin were visualized after fixation in formaldehyde (Hagan and Hyams, 1988) and using affinity-purified anti-Fus1 antibodies (1:20), affinity-purified anti-Sad1 antibodies (1:25; Hagan and Yanagida, 1995), or anti-γ tubulin (1:5; gift from M.J. Heitz) respectively. To enhance the GFP signal, anti-GFP antibodies were used (kindly provided by K. Sawin, Imperial Cancer Research Fund, London, United Kingdom), and the cells were scraped from MSA plates with a toothpick and smeared onto a coverslip, which was rapidly placed into methanol at −80°C. After 10 min at −80°C, cells were washed off in PEM buffer and processed as described (Hagan and Hyams, 1988). The advantages of fixing cells grown on solid MSA mating medium was that all stages of mating and sporulation were present in one sample. FITC-conjugated anti–rabbit, Cy3-conjugated anti–rabbit, or FITC-conjugated anti–mouse secondary antibodies were all from Sigma Chemical Co. (St. Louis, MO). To observe autonomous fluorescence of Fus1-GFP signals, cells were starved in MSL-N or on MSA, and were dried onto coverslips and inverted onto drops of glycerol containing 1 μg ml^{-1} diamidinophenylindole (DAPI).[1] Calcofluor was used for septum staining at 10 μg ml^{-1}. Color images were produced using a SIT Camera™ and a C2400 processor (Hamamatsu Phototonics, Bridgewater, NJ) for capturing the fluorescent signal into National Institutes of Health image software package on a MacIntosh Quadra 8500/100AV. A Pixel pipeline framegrabber was used to integrate 250 images to produce each single channel image, which were then merged in Adobe Photoshop.

Immunoblot Analysis

Crude protein extracts were prepared from 1×10^8 cells that had been

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1. *Abbreviations used in this paper*: DAPI, 4′,6-diamidino-2-phenylindole; FH, formin homology; GFP, green fluorescent protein; GST, glutathione S transferase; MSL, minimal sporulation media liquid; SPB, spindle pole body.

starved for nitrogen in MSL-N for 5 h. Cells were broken using glass beads in lysis buffer (50mM Tris-HCl, pH 7.5, 5mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1 mM PMSF, and 3.4 g/ml aprotinin) in a FASTprep™ FP120 machine (BIO 101 SAVANT; Savant Instrument Inc., Holbrook, NY) at max power for 15 s. Immunoblot analysis was performed according to Meloche et al. (1992) with two exceptions: transfer buffer 1 described in Harlow and Lane (1988) was used for electrotransfer of the proteins to the membranes, and proteins were detected with the Super Signal Ultra™ Western blotting detection reagent (Pierce Chemical Co., Rockford, IL).

Results

Actin Localization to the Projection Tip is Disrupted by Loss of Fus1 Function

Fission yeast F-actin cortical patches localize to the projection tip and the region just behind it during pheromone-induced polarization and cell fusion (Petersen et al., 1998). We have previously generated a strain in which the *fus1+* gene has been disrupted by insertion of *ura4+* at the nucleotide corresponding to amino acid residue 270 (Petersen et al., 1995). These cells are completely unable to fuse. Phalloidin staining of this disruption strain indicated that, while the actin cytoskeleton was polarized, it was often no longer associated with the very tip of the cells in 82% of prezygotes after 5 h in mating conditions (Fig. 1). Instead, the series of dots assumed a circular ring–like structure slightly behind the touching tips (data not shown). This altered pattern was also observed in the *fus1-B20* mutant (data not shown). In the minority of cells (18%), F-actin localized correctly to the tip. In cells completely deleted for *fus1* (EG999), this altered F-actin pattern was not observed. In these cells, F-actin dots were delocalized and seen all over the cytoplasm in 70% of the prezygotes (the remaining had F-actin localized correctly to the tip). Significantly, cells of the mutant, deletion, and disruption strain had clearly been able to form a projection tip. We interpret the data as indicating that F-actin is first correctly localized to the tip in all *fus1* mutants, but is then redistributed after a defective attempt to fuse. Thus, it is likely that Fus1 is required for the correct organization and stabilization of polarized F-actin at the tip, but is not
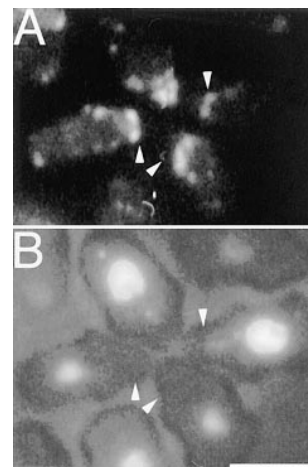

Figure 1. F-actin localization during the defective conjugation of a strain disrupted for the *fus1+* gene. The figure shows a paired panel showing F-actin stained with rhodamine-conjugated phalloidin (*A*) and the chromatin DAPI/phase contrast images (*B*) of the same cells. F-actin distribution in a *fus1*-disrupted strain (EG 439) was polarized. In the majority of the cells the F-actin staining was slightly back from the tips where the cells were touching (*arrows*). In the minority (18%), F-actin still localized to the very tip as it does in wild-type cells (see Fig. 4). Bar, 5 μm.

required to establish the initial polarization of the F-actin cytoskeleton.

Fus1 Localizes to the Projection Tip During Cell Fusion

We previously replaced the *fus1*⁺ gene with a mutated version of the gene that contained three copies of the hemagglutinin tag (HA) at its amino terminus (Petersen et al., 1995). Localization using the monoclonal antibody 12CA5 (Wilson et al., 1984) showed that Fus1 localized to the projection tips in conjugating cells (Petersen et al., 1995). This modified molecule was not able to support conjugation, which raised doubts as to whether its localization reflected that of the native molecule. We have therefore used two approaches to localize functional Fus1: raising antisera to the Fus1 protein, and tagging it with the green fluorescent protein (GFP) from *Aequoria victoria* (Heim et al., 1995).

Rabbit antibodies were raised against and affinity-purified with a bacterially produced fusion of the amino-terminal portion of Fus1 (residues 1–572) and GST. These antibodies recognized a single 160-kD band on an immunoblot (Fig. 2 *A*). The 160-kD band was only seen when the Fus1 molecule was overexpressed from a plasmid (pJP54: Pe-
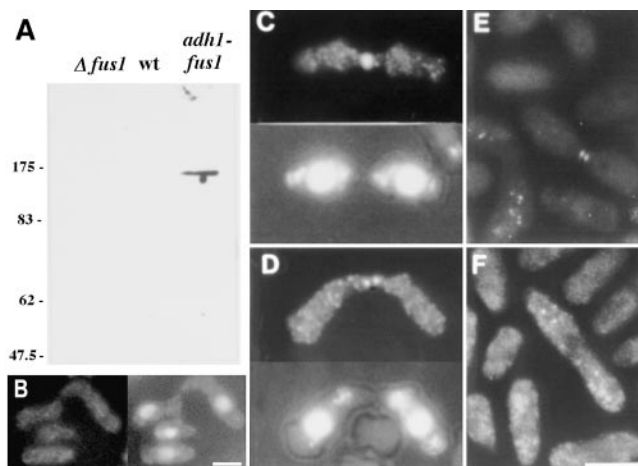

Figure 2. Different approaches reveal similar Fus1 localization patterns. Antibodies were raised to the first 571 aa of Fus1. After affinity purification these antibodies recognized a single band at ~160 kD in cells overexpressing Fus1 (*A,* lane *3*). No signal was seen in lanes containing extracts from *fus1*-deleted strains or wild-type cells (*A,* lanes *1* and *2*); the protein extract was made from conjugation cells. Localization of Fus1 with these antibodies in wild-type cells showed an association to the tips of conjugating cell pairs. *C* and *D* illustrate Fus1 localization (*top*) and DAPI/phase contrast (*bottom*) of the same cells. (*E*) Autonomous fluorescence of an integrated version of Fus1 bearing a carboxy-terminal GFP tag showed an identical localization pattern to that obtained with the antibodies; Fus1-GFP was associated with the tip at the point of contact of the conjugating pair, and after fusion cytoplasmic dots were seen. This localization of Fus1 to the tip was identical to that which we have reported before for an amino-terminal HA epitope-tagged molecule (Petersen et al., 1995). (*F*) Localization of Fus1 to the tip required cell–cell contact since an *h⁹⁰ mam2* strain failed to localize Fus1 to the tip. Instead, dispersed cytoplasmic dots were seen. (*B)* In a *fus1*-deleted strain (EG 999), no signal was seen using anti-Fus1 antibodies. Bar, 5 μm.

tersen et al., 1995), suggesting that Fus1 is present below the detection threshold of these antibodies in wild-type conjugating cells. By immunofluorescence, these antibodies stained a single dot at the very tip of each cell in wild-type pre-zygotes (Fig. 2, *C* and *D*). In addition, cytoplasmic dots were detectable before and after fusion, during karyogamy, and during horsetail movement (data not shown; horsetail movement is a stage that follows karyogamy, but precedes the first meiotic division during which the nucleus moves from one end of the cell to the other (Chikashige et al., 1994). The staining with the anti-Fus1 antibodies was not due to cross-reaction with an antigenically related molecule, because these antibodies failed to stain prezygotes that were attempting mating in the complete absence of a *fus1*⁺ gene (Fig. 2 *B*).

It is possible that the antibodies raised to the amino-terminal portion of the protein do not recognize all of the Fus1 molecules within the cell. This possibility could arise if the relevant epitopes were obscured by interactions with other proteins at discrete cellular locations. To rule out this possibility, the 3′ end of the *fus1*⁺ sequence was fused to sequences encoding GFP, and the subsequent construct was integrated into the genome so that it replaced the wild-type *fus1*⁺ coding sequences after the *fus1*⁺ promoter. The resultant fusion protein, Fus1-GFP, did not affect the mating efficiency, indicating that it retained full function (see Fig. 6 *A*). Localization of Fus1-GFP was slightly different from the immunofluorescence staining obtained with anti-Fus1 antibodies, since stained dots were still seen in meiosis I (data not shown, Fig. 2 *E*). Because this result indicates that Fus1-GFP is likely to be more sensitive than anti-Fus1 antibody staining, all subsequent localization experiments used GFP-fusions.

The fluorescent signal emitted by GFP depends upon cyclization of the molecule to generate the active fluorophore (Cubitt et al., 1995). Because cyclization can take some time, it was possible that we were not detecting all of the Fus1 GFP fusion protein. To this end we used anti-GFP antibodies to localize the fusion by indirect immunofluorescence. This approach has the added advantage that the sensitivity of detection should be considerably enhanced. The staining was identical to that seen with the autonomous fluorescence of GFP, with the sole exception that the dots persisted until sporulation (Fig. 3), when they were excluded from the forming spores.

Fus1 Colocalizes with F-actin at the Projection Tip

Given the similarity between the distribution of F-actin and Fus1, the observation that F-actin distribution is altered in cells lacking Fus1, and given the observed interaction between another formin and actin (Evangelista et al., 1997), we determined whether Fus1 and F-actin colocalize during conjugation by using video microscopy. While the initial spots of Fus1 staining in the main body of the cell occasionally colocalized with F-actin dots, the majority did not (Fig. 4 *A*.) At this stage of conjugation, F-actin was seen at the tips, while Fus1 was not. Once Fus1 appeared at the tip, the majority of the Fus1 colocalized with F-actin, but there were parts of the contact region that had Fus1 staining, but no F-actin staining (Fig. 4 *B*). After fusion, when Fus1 dots were again seen throughout the cytoplasm,
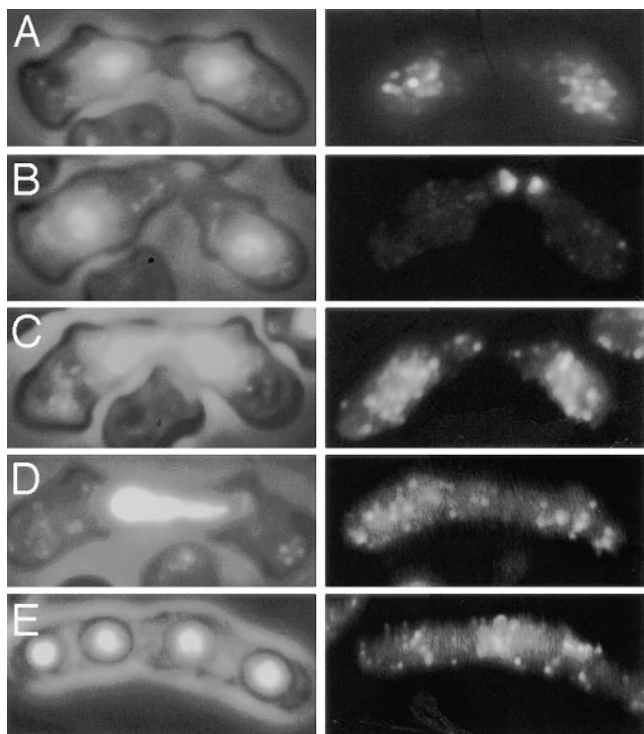
Figure 3. Localization of Fus1-GFP throughout conjugation and meiosis in the strain EG 938. The figure shows a series of panels containing anti-GFP immunofluorescence (*right*) and the chromatin DAPI/phase contrast (*left*) images of the same cells. Fus1-GFP was seen in the cytoplasm before fusion (*A*), and it localized to the tip in the conjugating pair (*B*). After fusion, the dots persisted from horsetail movements (*D*) to sporulation (*E*).
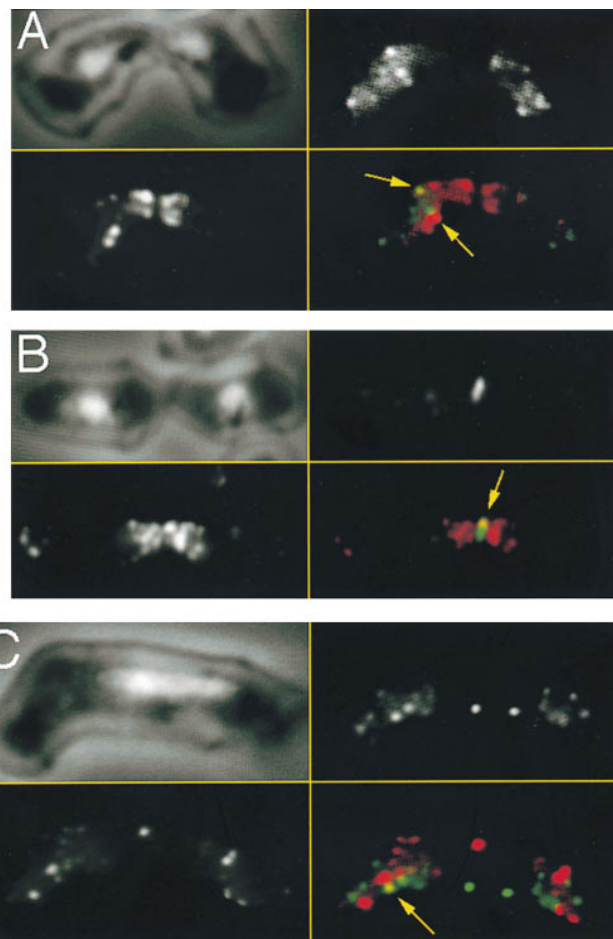

Figure 4. Superimposition of F-actin and Fus1 during conjugation. The figure shows a series of panels illustrating chromatin localization in DAPI/phase contrast images (*top left*), Fus1-GFP (*top right*), F-actin (*bottom left*), and false color images showing F-actin (*red*) and Fus1 (*green*; *bottom right*). Yellow arrows indicate colocalization of Fus1 and F-actin. (*A*) Before Fus1 located to the projection tips, only very few F-actin dots colocalized with Fus1. (*B*) At the projection tip before fusion, the majority, but not all, of Fus1 colocalized with F-actin, whereas later on during meiosis (*C*), again very few Fus1 dots were seen to colocalize with F-actin.

the dots on the whole did not colocalize with the F-actin dots. During meiosis, colocalization of the persistent Fus1, dots, and F-actin were extremely rare (Fig. 4 *C*). Thus, while some Fus1 does colocalize with F-actin before, during, and just after conjugation, the data suggests that Fus1 formed aggregates that can colocalize with F-actin, but do not always do so. The observation that F-actin localized to the tip before Fus1 (Fig. 4 *A*) is consistent with the ability of F-actin to localize to the tip in cells in which the $fus1^+$ gene had been disrupted (Fig 1 *A*) or deleted (not shown). To investigate this observation further, we counted the number of wild-type prezygotes that have Fus1 at their tips. We double-labeled a culture induced to conjugate with phalloidin and anti-Fus1 antibodies. All prezygotes have F-actin at their tips, whereas only 18% have Fus1. The later localization of Fus1 could indicate that Fus1 is localized to the tips following a discrete interval after commitment to polarized growth, or at a point after contact with an active mating partner.

Cell–Cell Contact is Required to Localize Fus1 to the Projection Tip

The observation of Fus1 dots in the cytoplasm before fusion (Fig. 3) suggests that reorganization of Fus1 localization occurs during mating. To test whether Fus1 localization may require an active partner, we looked at Fus1-GFP localization in an h^{90} *mam2* strain. The h^{90} mating type means that

this strain is homothallic, and frequently undergoes mating type switches. The result is a population with both mating types. The $mam2^+$ gene encodes the receptor for P-factor, and is expressed only in *M* cells. Therefore, in a h^{90} *mam2* strain, the *P* cells will attempt mating, but the *M* cells will be unable to respond due to the lack of the P-factor receptor, and so the cells will fail to initiate fusion. Under these conditions, Fus1-GFP was never seen at the cell tips, but it appeared as cytoplasmic dots in single cells whose morphology indicated that they were responding to M-factor (Fig. 2 *F*). These data were confirmed by the lack of Fus1-GFP localization in heterothallic h^+ cells treated with M-factor to induce hyperpolarization in the absence of a mating partner (data not shown). These two observations indicate that Fus1 localization to the projection tip requires contact with an active mating partner.
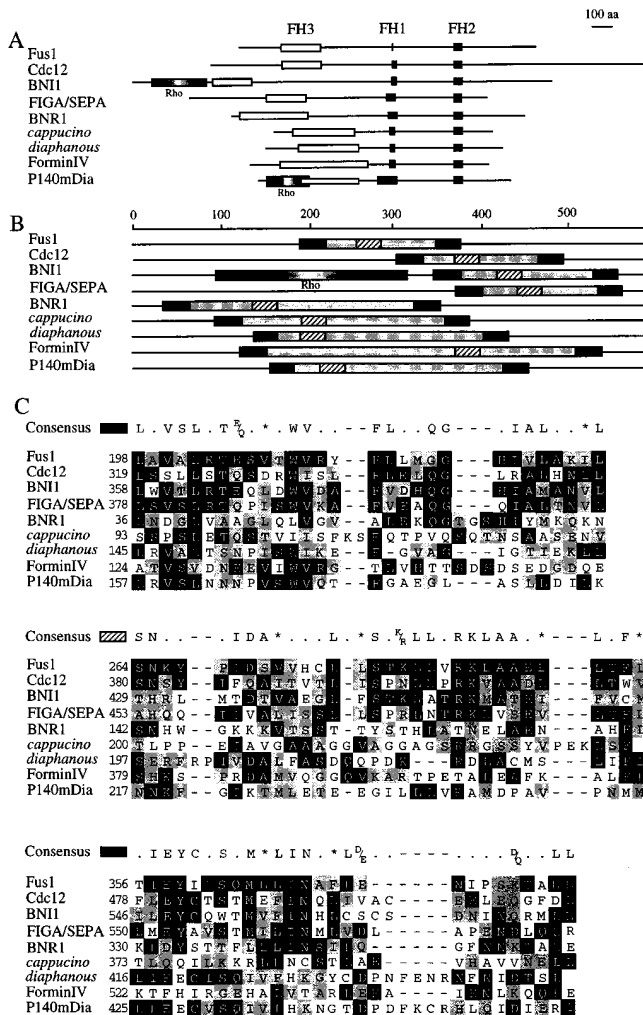
Figure 5. A third region of homology FH3 is found in the amino termini of formins. (*A*) A cartoon depicting the structure of the nine formin homologs identified to date. The relative positions of the FH1 (*black boxes*), FH2 (*grey boxes*), the rho-binding region (*Rho*), and FH3 (*open boxes*) domains are indicated. (*B*) The FH3 region is a tripartite block of similarity with the same relative order in each formin. The size of this region and position relative to the amino termini is shown. (*C*) Similarity of the three blocks within the FH3 region aligned by the multiple alignment function of Lasergene™ (DNASTAR Inc.). Identical residues at the same position are black, and similar amino acids are grey. An asterisk in the consensus sequence indicates hydrophobic amino acids. A minimum of four identical amino acids was required to give a consensus sequence. The position of the three blocks relative to the amino termini is shown on the left side of the cartoon.

The FH3 Domain: Fus1 and Other Formins Share a Previously Unidentified Region of Homology

Two regions of homology have been described in the carboxy termini of formins: FH1 and FH2 (formin homology regions 1 and 2; Castrillon and Wasserman, 1994). Motivated by small but consistent homology islands in dot-plot comparisons of Fus1 with other formins, the sequences that lie between the initiator ATG codon and the start of the FH1 domain of all formins were aligned individually to Fus1 using the GCG program Eclustalw (Genetics Com-
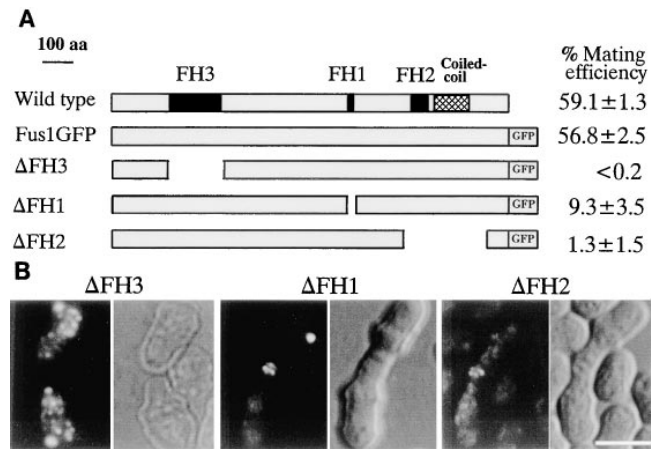

Figure 6. The consequences of deletion of the three different FH domain from the genomic copy of *fus1*. The resultant mutants were simultaneously tagged with GFP at their carboxy termini in order to determine the effect of the deletion upon localization of Fus1. (*A*) Deletion of any of the three FH regions resulted in dramatically reduced mating efficiencies when compared with a wild-type control strain and a control strain in which the wild-type Fus1 molecule had been tagged at its carboxy terminus with GFP. The mating efficiency was calculated according to Petersen et al. (1995; $n = 500$). (*B*) GFP autonomous fluorescence showed that fusion proteins lacking FH1, or the region comprising FH2 and the coiled coil, still localized to the projection tip. The figures below represent Normaski image of the same cells. In contrast, anti-GFP immunofluorescence showed that deletion of FH3 abolished Fus1-GFP localization. To the right of this panel, a phase contrast image of the same cell is shown. Bar, 5 μm.

puter Group, Madison, WI). Based on the information from the dot plot analyses and these secondary alignments, three potential homology regions were identified. These regions were then finally aligned using the Lasergene™ multiple alignment function (DNASTAR Inc., Madison, WI) with final minor adjustments by eye. From these analyses, it was apparent that there is a third FH region near the amino termini of formins. This region consists of three blocks of similarity in the same relative order in each formin (Fig. 5). The first part of the first FH3 block and the whole of the third block shows the highest level of conservation. The sequence identity, spacing of the sequence blocks, and the similarity at the amino acid level suggest that the fungal sequences form a distinct subgroup with higher similarity than the other members. In line with preceding nomenclature, we call this region FH3. The FH3 domain of formins is the most variant of the homology regions, which probably explains why it has eluded identification to date. The FH3 domain in Bni1p is distinct from the region that interacts with Rho1. The algorithm of Lupas et al. (1991) predicted an additional feature in the Fus1 sequence: a coiled-coil motif between residues 1145 and 1240 (Fig. 6 *A*). Similar regions of coiled coil have been described in the carboxy terminus of Cdc12 and SEPA (Chang et al., 1997; Harris et al., 1997). Furthermore, we have found a stretch predicted to form a coiled-coil structure in the comparable region of *S. cerevisiae* Bni1p (data not shown).

The Fus1 FH3, but not FH1 or FH2 Domains, is Required for Tip Localization

To examine the function of the three different FH domains in Fus1, we deleted each in turn from the genomic copy of *fus1*, which was simultaneously tagged with GFP at its carboxy terminus in a homothallic strain (Fig. 6 *A*). The dramatically reduced mating efficiencies of all three strains showed that each of these domains is required for conjugation (Fig. 6 *A*). Fusion proteins that lacked FH1, or the region comprising FH2 and the coiled-coil, still localized to the projection tip (Fig. 6 *B*, Table III). In fact, more cells had staining at their tips than wild-type cells, probably indicating a prolonged attempt to conjugate, after which the protein delocalizes. In contrast to the localization of Fus1 ΔFH1 and Fus1 ΔFH2 to the tips, deletion of FH3 abolished Fus1-GFP localization (Fig. 6 *B,* Table III). The requirement for FH3 for tip localization of Fus1 is consistent with the stronger effect on the mating efficiency seen in the FH3 deleted strain (Fig. 6 *A*). Similar to *fus1.B20* (Egel, 1973a), the two cells attempting conjugation in strains lacking the FH1 and FH2 domains often had flat ends rather than the rounded points seen in FH3 and *fus1* deletants attempting conjugation. This observation may suggest that cell wall expansion, but not breakdown, can occur in the former class of mutants. Alternatively, it may reflect the leakier nature of the block in these mutants. These data strongly suggest that the FH3 domain binds to the cortex, while FH1 and FH2 are required to mediate other essential interactions of Fus1.

A potential role for the FH1 domain has been suggested by the ability of the proline-rich FH1 domains of formins to interact with profilin (Evangelista et al., 1997; Jansen et al., 1996; Chang et al., 1997; Imamura et al., 1997). In a separate study we have shown that fission yeast profilin, which is encoded by the *cdc3+* gene, is required for conjugation (Petersen et al, 1998). Therefore, it was possible that the FH1 domain of Fus1 could interact with Cdc3. If this were the case, Cdc3 localization could be disrupted by deletion of the FH1 domain. However, Cdc3 tip association was unaffected by removing the FH1 domain, indicating an alternative mechanism for Cdc3 localization (Fig. 7 *A*). Disruption of Fus1 resulted in a Cdc3 staining pattern similar to that of F-actin, as rings were seen just back from the tips in 78% of the prezygotes (data not shown). Fus1-GFP localized normally in the temperature-sensitive *cdc3.124* mutant attempting mating at the restrictive temperature of 32°C (Fig. 7 *B*).
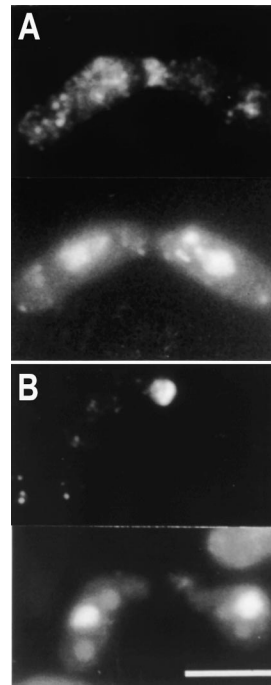
Figure 7. Cdc3 localizes to the tip independently of the Fus1 FH1 domain. (*A*) The figure shows anti-Cdc3 immunofluorescence (*top*) and chromatin DAPI/phase contrast images (*bottom*) of the same cell. Cdc3 still localized to the tip in the strain in which the FH1 domain of Fus1 has been deleted. (*B*) Fus1-GFP localized normally in the *cdc3.124* mutant, only the *h−* strain in this cross contains the GFP-tagged Fus1 protein, and therefore, a signal is only seen in one of the two mating partners. Bar, 5μm.

The Fus1 FH3 Domain Directs a GFP Fusion Protein to the Projection Tip

The requirement for the FH3 domain to localize Fus1 to the conjugation tip led to the prediction that overexpressing this domain may facilitate competition for binding the wild-type molecule to the target protein that normally binds this domain at the tip. This competition would be expected to produce a fus− phenotype with cells accumulating as prezygotes. To test this possibility, the FH3 region from Fus1 was placed in a multicopy plasmid under control of the thiamine-repressible *nmt1+* promoter (Maundrell, 1993). Expression of Fus1 FH3 domain and starvation of the strain were induced in a homothallic strain by growth on solid MSA sporulation medium lacking thiamine. 19% of the zygotes had a fus− phenotype (Fig. 8 *A*; Table IV), suggesting that expression of the FH3 domain interfered with normal Fus1 function.

We next asked whether the FH3 domain alone was sufficient to direct localization of a chimeric molecule to the tip by expressing FH3-GFP fusions in mating cells from a plasmid under control of the thiamine-repressible *nmt1+* promoter (Maundrell, 1993). The fusion protein localized to the projection tip in 21% of the prezygotes having a GFP signal, and to cytoplasmic dots (Fig. 8 *B*, Fig. 9 *A*). This result is in remarkable agreement with the frequency of Fus1 tip localization (18%) during conjugation (Table III; 4-h time point). FH3-GFP localization to the projection tips is also seen in *fus1*-deleted strains (Fig 9 *B*). These data are consistent with the FH3 domain being responsible for directing Fus1 to the projection tip.

Evidence that the FH3 Domain is a General Targeting Motif

When the FH3 domain was expressed in the vegetative cells in the mating assays, some of these cells showed defects in cytokinesis and nuclear positioning (data not shown). As
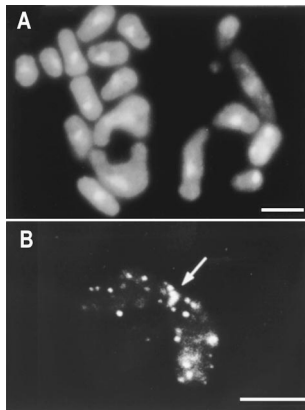
Table III. The Frequency of Fus1 Tip Localization in Prezygotes of Various Strains

Strain	Hours of nitrogen starvation			
	4	5	6	7
Wild type (anti-Fus 1) (%)	18	20	16	ND
Fus 1-GFP (%)	23	22	19	ND
Fus 1-GFP ΔFH1 (%)	26	49	34	22
Fus 1-GFP ΔFH2 (%)	20	35	18	4
Fus 1-GFP ΔFH3 (%)	<0.2	<0.2	<0.2	<0.2
Fus 1 FH3-GFP (%)	21	23	14	ND

ND, not detected.

Figure 8. Overexpression of the Fus1 FH3 region interfered with normal Fus1 function. (*A*) When the Fus1 FH3 region was placed under control of the thiamine-repressible promoter in a multicopy plasmid, induction of expression in a nitrogen-starved wild-type strain gave rise to fus⁻ cells which were unable to conjugate. (*B*) Fusion of the Fus1 FH3 domain to GFP showed an association of the fusion protein to the tip (*arrow*), along with cytoplasmic dots. This result is consistent with the FH3 domain being required for Fus1 localization to the tip (Fig. 6 *B*). Bar, 5 μm.
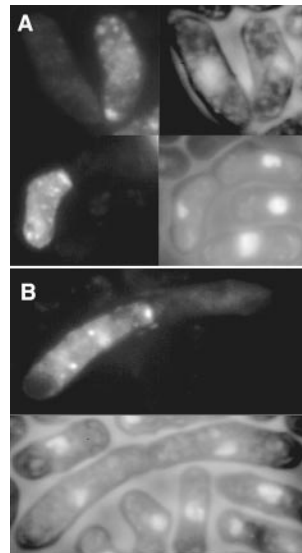

Figure 9. The Fus1 FH3-GFP fusion localizes to the projection tip and the nucleus in a Fus1-deleted strain. The figure shows GFP autonomous fluorescence (*A, left*; *B, top*) and chromatin DAPI/phase contrast images (*A right*; *B, bottom*) of the same cells. (*A*) In wild-type cells, the Fus1 FH3-GFP fusion localizes to the projection tip. Only one cell in these mating pairs shows a GFP signal, indicating that the localization signal is dependent upon the level of expressed fusion protein. (*B*) In a Fus1-deleted strain, the fusion protein localizes to the projection tip as well. In addition, a dot is also seen on the nucleus, and it is very likely be the SPB (see text).

these defects are associated with features of mitotic division rather than sexual differentiation, they raised the possibility that, in addition to affecting conjugation, the expression of Fus1 FH3 may interfere with some aspects of mitotic growth. Therefore, the culture was spotted onto plates that favor mitotic growth (AA media) with (gene off), or without (gene on) thiamine. The normal growth of cells under repressing conditions was perturbed by FH3 induction (Fig. 10 *A*). Many cells failed to maintain the normal central location of their nuclei, and septa and cytokinesis was clearly defective in some cases (Fig. 10, *C–E*; Table IV). The normal central location of the interphase nucleus is maintained by spindle pole body (SPB)-mediated microtubule interactions (Hagan and Yanagida, 1997). However, microtubule distribution was unaffected by Fus1 FH3 expression (data not shown). Thus, the data are consistent with defective SPB and cytokinetic ring function.

The effect of overexpressing the FH3 domain of Fus1 on cytokinesis was surprising, because strains that lack the *fus1⁺* gene grow and divide normally, and the gene is not transcribed during vegetative growth (Petersen et al., 1995). Therefore, no normal role in the mitotic cell cycle was expected. This result suggested that the Fus1 FH3 domain was inappropriately interacting with an FH3-binding protein at the SPB, the cytokinetic ring, or in the cytoplasm to

Table IV. Expression of FH3 Domains Affects Cell Fusion, Nuclear Positioning, and Cytokinesis

| | Phenotype | | | |
| | ![mating cells] | ![nuclear] | ![septum] | ![septation] |
Strain	Mating cells with fus⁻ phenotypes	Nuclear position defects	Septum position defects	Septation defects
	%	%	%	%
h^{90} + vector	0.8	5	1	0.6
h^{90} + Fus1 FH3	19	17	15	6
h^{90} + Fus1 FH3-GFP	11	12	11	3
h^{90} + Cdc12 FH3-GFP	9	13	11	4

interfere with normal targeting of other proteins to the SPB and the actin ring. Localization of Fus1 FH3-GFP showed the former to be the case. Several cytoplasmic dots with varying intensities were seen at all stages of the cell cycle, and occasional dividing cells with weak equatorial ring staining were seen (*arrow heads,* Fig. 10 *B*). Some cytoplasmic dots were much stronger than the others (*arrows,* Fig. 10 *B*). Significantly, no tip staining was observed during vegetative growth, indicating that FH3 tip association is restricted to conjugation. Staining Fus1 FH3-expressing cells with antibodies to the SPB component Sad1 (Hagan and Yanagida, 1995) and with antibodies to γ-tubulin (M. Heitz and I.M. Hagan, unpublished data) showed that, in more than half of the late G2 cells with a GFP signal above background, Fus1 FH3 colocalized with the γ-tubulin at the SPB (Fig. 11 *B*). In septated binucleate cells and cells in early G2 phase, colocalization was seen in up to a fifth of the cells with a GFP signal. Staining was notably different with anti-Sad1 antibodies. Cells with a strong nucleus-associated GFP dot failed to stain with anti-Sad1 antibodies, while cells with no GFP signal had a normal Sad1 signal (Fig. 11 *A*). This result raised the intriguing possibility that the overexpressed Fus1 FH3-GFP was blocking anti-Sad1 antibody binding either by directly binding to Sad1, or to a Sad1-containing complex.

Moderate overexpression of Sad1 protein results in its localization to the nuclear periphery (Hagan and Yanagida, 1995). A corresponding alteration in the distribution of FH3-GFP in cells with increased Sad1 levels would indicate some form of an interaction between Sad1, or a Sad1 containing complex and Fus1 FH3-GFP. In Sad1-overexpressing cells, Fus1 FH3-GFP localization did indeed follow Sad1 distribution to the nuclear periphery in 30% of the cells that had a GFP signal (Fig. 11 *D*).

To determine whether the localization to these structures was specific to the FH3 domain of Fus1, or whether other fission yeast FH3 domains would direct GFP to similar structures, the FH3 domain of Cdc12 was fused to GFP and localized in logarithmically growing cells, either with or without a *sad1⁺*-bearing plasmid. Overexpression of
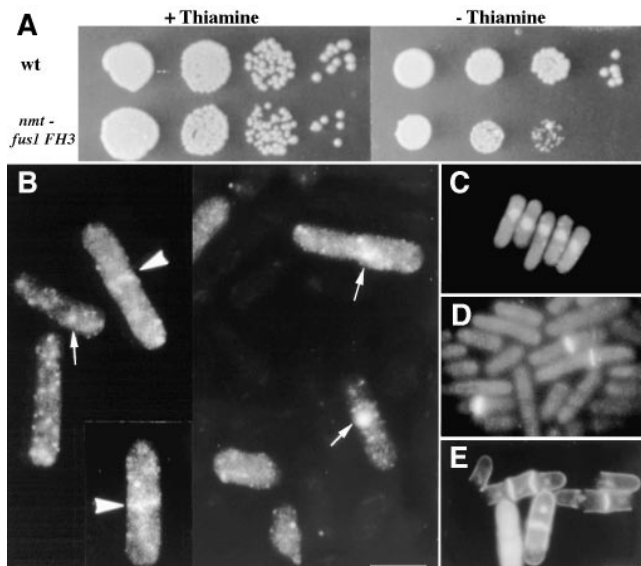
Figure 10. Overexpression of Fus1 FH3 interfered with mitotic growth. (*A*) Serial dilutions of each culture were spotted onto solid AA media with and without thiamine. In contrast to normal growth under repressing conditions, induction of Fus1 FH3 perturbed growth. DAPI and calcofluor staining of these cells shows that: (*C*) nuclei were not always in their normal central position; (*D*) the septa were often off center, and cytokinesis was not always completed; and (*E*) cells with more than one septum were seen. (*B*) Localization of Fus1 FH3-GFP after 15 h of induction on solid AA media was seen as several cytoplasmic dots with varying intensities. In some cases one of the cytoplasmic dots was stronger than the others (*arrows*), and some cells also had weak equatorial ring staining (*arrowheads*). Bar, 5 μm.

Cdc12 FH3-GFP from the *nmt1*[+] promoter on AA media without thiamine resulted in similar defects to those seen with expression of Fus1 FH3. In many cases the septum or the nucleus was misplaced from the center of the cell, and cytokinesis was not always completed (Fig 11 *F*; Table IV). In wild-type cells, the Cdc12 FH3-GFP fusion protein colocalized with γ tubulin in 46% of late G2 cells having a GFP signal (Fig. 11 *C*), and in 15% of binucleate cells and cells in early G2. In Sad1-overexpressing cells, the fusion protein went to the nuclear periphery in 25% of cells that had a GFP signal (Fig. 11 *E*).

These data show that FH3 domains are capable of directing the localization of chimeric fusion proteins to discrete structures within the cell, and are consistent with the presence of FH3 domain interacting protein(s) at multiple locations.

Discussion

Formins are emerging as key molecules in the execution of a number of different morphogenic events (Marhoul and Adams, 1995; Woychik et al., 1990; Castrillon and Wasserman, 1994; Emmons et al., 1995; Evangelista et al., 1997; Imamura et al., 1997; Chang et al., 1997; Harris et al., 1997). Here we report an analysis of the fission yeast formin in Fus1. One of the major advantages of studying this formin is that it is not essential for vegetative growth.
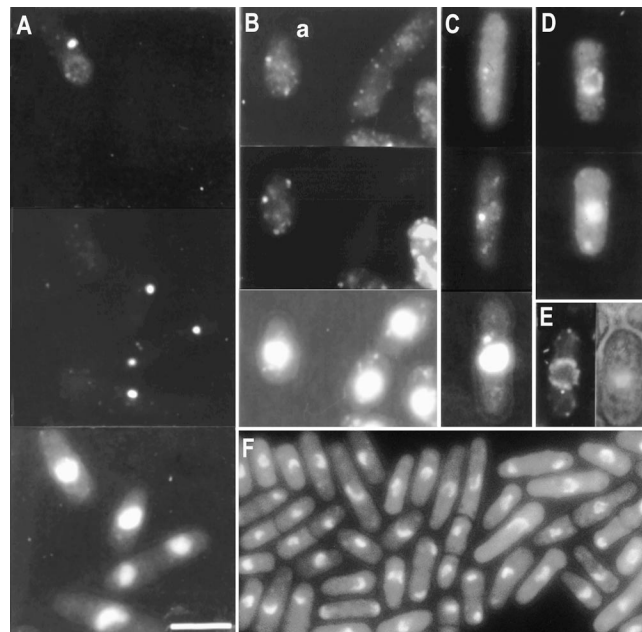

Figure 11. Fus1 FH3-GFP and Cdc12 FH3-GFP colocalized with γ tubulin at the spindle pole body. This localization was altered when the SPB component Sad1 was driven to the nuclear periphery by overexpression. (*A*) The figure shows Fus1 FH3-GFP autonomous fluorescence (*top*), anti-Sad1 immunofluorescence (*middle*), and the chromatin DAPI/phase contrast (*bottom*) images of the same cells. In cells with strong nuclear-associated Fus1 FH3-GFP dots, anti-Sad1 antibodies fail to stain the SPB. All SPBs were in the same focal plane. (*B*) The figure shows a series of panels illustrating anti-γ tubulin immunofluorescence (*top*), GFP autonomous fluorescence (*middle*), and the chromatin DAPI/phase contrast (*bottom*) images of the same cells. The strong Fus1 FH3-GFP signal (*a*) colocalized with γ tubulin at the SPB. Thus, unlike Sad1 detection, the ability to detect γ tubulin with γ tubulin antibodies was not blocked by the presence of the FH3GFP under identical conditions. (*C*) The figure shows a series of panels illustrating GFP autonomous fluorescence (*top*), anti-γ tubulin immunofluorescence (*middle*), and the chromatin DAPI/phase contrast (*bottom*) images of the same cells. The strong Cdc12 FH3-GFP signal colocalized with γ tubulin at the SPB. (*D*) In cells overexpressing the SPB component, Sad1 Fus1 FH3-GFP localization was seen as a ring around the nuclear periphery. The figure shows GFP autonomous fluorescence (*top*) and the chromatin DAPI/phase contrast (*bottom*) images of the same cells. (*E*) Overexpression of Sad1 drove Cdc12 FH3-GFP around the nuclear periphery. The figure shows GFP autonomous fluorescence (*left*) and the chromatin DAPI/phase contrast (*right*) images of the same cell. (*F*) Overexpression of Cdc12 FH3-GFP affected mitotic growth, positioning of the interphase nucleus and septum, and the completion of cytokinesis. Bar, 5 μm.

Fus1 is only produced upon commitment to mating, whereupon it is required for cell fusion (Petersen et al., 1995). We have therefore been able to mutate the gene in various ways at its native locus, and to determine the consequences of these manipulations upon induction of the mating response.

The Mechanism of Fus1 Tip Localization in S. pombe

Once polarized cell growth has brought two mating partners into contact, the cell walls are degraded at the contact

point in a highly localized and regulated fashion to enable the two genomes to fuse after karyogamy. Fus1 is required for this cell wall degradation process (Petersen et al., 1995). We have consistently localized Fus1 to the fusion point by three different approaches: tagging at the amino terminus with the HA-tag, carboxy-terminal tagging with GFP, and immunofluorescence with anti-Fus1 antibodies.

F-actin associates with the cell tip during growth towards the pheromone source that is produced by prospective mating partners (Petersen et al., 1998). This association with the projection tip is before and independent of Fus1 localization to the tip (Fig. 4 *A*); however, the phenotype of the *fus1*[+] deletion and disruption strains indicated that Fus1 is required to stabilize or mediate F-actin association with the fusion zone after cell–cell contact. F-actin was rarely seen at the tip in cells in which the *fus1*[+] gene had been disrupted by the insertion of the *ura4*[+] gene at the nucleotide corresponding to amino acid residue 270. Instead, F-actin was seen slightly back along the projection tubes of the majority of prezygotes in this strain. When the orientation of the specimen was appropriate, the F-actin dots at this stage were often arranged in a circular fashion reminiscent of a ring. While the lack of conjugation showed that the gene disruption had blocked the production of full-length Fus1 protein, it is highly likely that some portion of the protein was produced, as complete deletion of *fus1*[+] resulted in random cytoplasmic distribution of F-actin dots in the prezygotes after an initial association with the tip. It would therefore seem likely that after cell–cell contact, the role of the actin cytoskeleton is to expand the cell wall at the point of fusion, and that this expansion is regulated by dilation of an F-actin ring between the two cells in the newly formed zygote. In the disruption strain the ring remains intact, associated with the cortex, still expands, and so moves back along the projection tip.

Multiple Commitment Points During Conjugation

The two different roles played by the actin cytoskeleton during mating—polarized cell growth and coordinated cell wall degradation—underline the importance of correctly coordinating fusion events. Cell–cell contact is required before Fus1 is localized to the projection tip. Thus, two decision points are defined: commitment to polarized growth and the attachment to a mating partner, which stimulates the recruitment of Fus1 to the tip and chromatin rearrangements within the nucleus (Chikashiga et al., 1997). Recruitment of Fus1 may be involved in a feedback loop to signal the cessation of tip growth, as cells that harbor the *fus1.B20* mutant continue to elongate when fusion is defective (Petersen et al., 1995). Alternatively, this feedback loop may be activated by cytoplasmic mixing after cell fusion. In this case, *fus1* mutants continue to elongate because the cytoplasms do not mix.

How Does the Cell Sense the Binding of a Partner?

It is possible that cell–cell contact is registered by localized pheromone gradients through the established signal transduction pathway (reviewed by Nielsen and Davey, 1995). If a pheromone gradient is important, the high levels required to generate a signal have to be localized at the point of cell–cell contact. In this case, adding a pheromone to a heterothallic strain fails to induce Fus1 localization and heterochromatin rearrangements (Chikashiga et al., 1994) because the same level of pheromone is registered all around the cell (Fig. 2). Alternatively, there may be a distinct receptor-mediated signal transduction pathway that is activated after cell–cell contact-specific agglutination at the projection tips. A potential candidate for such a pathway may involve the MAP kinase Spm1 (Zaitsevskaya-Carter and Cooper, 1997). Disruption of *spm1* interferes with cytokinesis and morphogenesis, and greatly perturbs conjugation.

FH3-mediated Fus1 Binding to the Tip

Two formin homology regions have been described previously (FH1 and FH2), and a domain that can interact with members of the rho family of small GTP binding proteins has been described in two of the formins: Bni1p and p140mDia. We describe an additional amino-terminal tripartite formin homology region that we call FH3 (Fig. 5). The similarity between FH3 domains is strongest in comparisons of different fungal members. Different parts of the sequence are conserved to varying degrees in the metazoan family members. The FH3, but not the FH1 or FH2 domain of Fus1, was required to direct Fus1 localization to the tip. Since deletion of all three domains drastically reduces mating efficiency, FH1 and FH2 are presumably required for some other aspects of Fus1 function.

The ability of the FH3 domain to direct a GFP fusion protein to the projection tip and compete with native Fus1 at this site, thus generating a fus[−] phenotype with cells accumulating as prezygote pairs, suggests that it alone is sufficient to locate Fus1 to the tip. However, other parts of the protein are likely to stabilize or enhance the binding to the tip (see below). The Fus1 FH3 domain can also compete with additional proteins required for other events in the life cycle when Fus1 is not normally present. Thus, Fus1 FH3 targets the fusion protein to the SPB and the equatorial ring. This targeting to new locations leads to defects in nuclear positioning and cytokinesis (Fig. 10). Considering the similarity between this localization pattern and that reported for Cdc12 (Chang et al., 1997) and the concomitant cytokinesis defects, it is possible that the Fus1 FH3 domain is affecting cytokinesis by binding to the normal partner of the FH3 domain of Cdc12. Consistently, we found that the FH3 domain of Cdc12 behaved like that of Fus1; it colocalized with the SPB marker γ tubulin and followed Sad1 to the nuclear periphery when it was driven there by moderate overexpression (Hagan and Yanagida, 1995). The block to anti-Sad1 antibody binding to the Sad1 protein that results from expression of the Fus1 FH3-GFP fusion suggests that epitope masking of Sad1 is occurring for some reason. Whether or not the epitope masking is due to a direct interaction with Sad1 is not clear, but this finer point does not detract from the fact that the FH3 domain is never seen around the nuclear periphery in wild-type cells, but is in strains overexpressing Sad1. Thus, the FH3 domain is capable of directing location to more than one site within the cell.

The Fus1 FH1 Domain

It has been suggested that the proline-rich formin homol-

ogy domain, FH1, is responsible for the association of formins with the actin-binding protein profilin in vivo. This suggestion was stimulated by the demonstration that profilin binds to polyproline regions in in vitro assays (Tanaka and Shibata, 1985). Several reports describe an in vitro interaction between profilin and the proline-rich FH1 domain of formins (Chang et al., 1997; Evangelista et al., 1997; Imamura et al., 1997), and some use two hybrid and synthetic lethality data to argue for the same interaction in vivo. While the fission yeast profilin homolog Cdc3 is absolutely required for conjugation (Petersen et al., 1998), it localizes to the tip independently of the FH1 domain of Fus1. We have also determined that Fus1-GFP localizes to the tip in a *cdc3.124* mutant at a temperature that is restrictive for both conjugation and mitotic growth, suggesting that the converse may be true, and that Fus1 may localize independently of Cdc3 function. One potential problem with this conclusion, however, is that we cannot rule out the possibility that a Fus1-interacting function of Cdc3 is unaffected at this restrictive temperature, and that it is some other function of this multifunctional protein that is temperature-sensitive in *cdc3.124*. However, it is clear that Fus1 localizes to the tip when its FH1 domain has been completely removed. Finally, attempts to detect any interaction between Fus1 and Cdc3 by immunoprecipitation have failed, and we have detected only weak interactions between the FH1 domain of Fus1 and Cdc3 in the budding yeast two-hybrid assay (as would be expected for a proline-rich sequence; J. Petersen, unpublished data). It is perhaps important in assessing this body of evidence that argues against an interaction between the Fus1 FH1 domain and Cdc3 to note that the FH1 domains of the other formins contain far more prolines than does the FH1 domain of Fus1, and that profilin binding requires runs of 8–10 prolines (Sohn and Goldschmidt-Clermont, 1994). If the FH1 domain of Fus1 does not bind to profilin, it may confer the ability to interact with SH3 or WW domains in target molecules (Sudol, 1996). Profilin may therefore execute its role in conjugation independently of the Fus1 FH1 domain.

Multiprotein Complex at the Tip

Formins interact with molecules such as actin and actin-binding proteins (Imamura et al., 1997; Evangalista et al., 1997), suggesting that further work may well identify large complexes containing Fus1. Two pieces of data suggest that Fus1 may interact with multiple partners. The first is that while deletion of the FH1 or FH2 domains severely reduces conjugation efficiency, it does not reduce it to the zero level seen with deletion of either FH3 or the entire molecule. This fact suggests that although the functions of FH1 and FH2 are virtually essential, the molecules with which they interact must be able to bind to at least one other partner or other regions of Fus1. Thus, in the absence of interaction of the domain with Fus1, some activity of the overall complex is achieved. Clearly, if the complex were unable to localize at all, as is the case for Fus1 which lacks the FH3 domain, its function would be completely lost, as we have seen. Precedents for such a situation include the interaction between the budding yeast SPB components Cdc31p and Kar1p. Both are essential. Kar1p is required to localize Cdc31p to the SPB, but in extragenic

suppressors the requirement for Kar1p binding is bypassed, suggesting that the complex contains more than just Kar1p and Cdc31p (Biggins and Rose, 1984; Vallen et al., 1994). Thus, extragenic suppressors of FH1 or FH2 deletions could be expected to identify other components of the complex. Complexes are also suggested by the large aggregates of Fus1 seen before and after conjugation.

The identification of the FH3 domain and its ability to localize in a similar way to, and compete with, the full-length molecule suggest that this is a functionally relevant motif. The ability of Cdc12 FH3 domain to target GFP to discrete locations suggests that the FH3 motif in other formins will similarly target them to discrete locations. We have shown that the ability to manipulate formins that are absolutely required for an inducible event offers a key to unravelling the functional complexities of this expanding family of complex molecules.

References

Adams, A.E.M., D.I. Johnson, R.M. Longnecker, B.F. Sloat, and J.R. Pringle. 1990. *CDC42* and *CDC43*, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae. J. Cell Biol.* 111:131–142.

Ayscough, K.R., J. Stryker, N. Pokala, M. Sanders. P. Crews, and D.G. Drubin. 1997. High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor Latrunculin-A. *J. Cell Biol.* 137:399–416.

Balasubramanian, M.K., B.R. Hirani, J.D. Burke, and K. Gould. 1994. The *Schizosaccharomyces pombe cdc3*+ gene encodes a profilin essential for cytokinesis. *J. Cell Biol.* 125:1289–1301.

Biggins, S., and M.D. Rose. 1984. Direct interactions between yeast spindle pole body components: Kar1p is required for Cdc31p localization to the spindle pole body. *J. Cell Biol.* 125:843–852.

Bresch, C., G. Müller, and R. Egel. 1968. Genes involved in meiosis and sporulation of a yeast. *Mol. Gen. Genet.* 102:301–306.

Bretscher, A., B. Dress, E. Harsay, D. Schott, and T. Wang. 1994. What are the basic functions of microfilaments? Insights from studies in budding yeast. *J. Cell Biol.* 126:821–825.

Castrillon, D.H., and S.A. Wasserman. 1994. *Diaphanous* is required for cytokinesis in *Drosophila* and shares domains of similarity with the products of the *limb deformity* gene. *Development.* 120:3367–3377.

Chang, F., D. Drubin, and P. Nurse. 1997. Cdc12p, a protein required for cytokinesis in fission yeast, is a component of the cell division ring and interacts with profilin. *J. Cell Biol.* 137:169–182.

Chant, J. 1996. Generation of cell polarity in yeast. *Curr. Opin. Cell Biol.* 8:557–565.

Chikashige, Y., D.-Q. Ding, H. Funabiki, T. Haraguchi, S. Mashiko, M. Yanagida, and Y. Hiraoka. 1994. Telomere-led pre-meiotic chromsome movement in fission yeast. *Science.* 264:270–273.

Chikashige, Y., D.-Q. Ding, Y. Imai, M. Yamamoto, T. Haraguchi, and Y. Hiraoka. 1997. Meiotic nuclear reorganization: switching the position of centromeres and telomeres in fission yeast *Schizosaccharomyces pombe. EMBO (Eur. Mol. Biol. Organ.) J.* 16:193–202.

Cubitt, A.B., R. Heim, S.R. Adams, A.E. Boyd, L.A. Gross, and R.Y. Tsien. 1995. Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* 20:448–455.

Doyle, T., and D. Botstein. 1996. Movement of yeast cortical actin cytoskeleton visualized in vivo. *Proc. Natl. Acad. Sci. USA.* 93:3886–3891.

Egel, R. 1973a. Commitment to meiosis in fission yeast. *Mol. Gen. Genet.* 121:277–284.

Egel, R. 1973b. Genes involved in mating type expression of fission yeast. *Mol. Gen. Genet.* 122:339–343.

Egel, R., M. Willer, S. Kjœrulff, J. Davey, and O. Nielsen. 1994. Assessment of

pheromone production and response in fission yeast by a halo test of induced sporulation. *Yeast*. 10:1347–1354.

Emmons, S., H. Phan, J. Calley, W. Chen, B. James, and L. Manseau. 1995. *Cappuccino*, a *Drosophila* maternal effect gene required for polarity of the egg and embryo, is related to the vertebrate *limb deformity* locus. *Genes Dev*. 137:169–182.

Evangelista, M., K. Blundell, M.S. Longtine, C.J. Chow, N. Adams, J.R. Pringle, M. Peter, and C. Boone. 1997. Bni1p, a yeast formin linking Cdc42p and the actin cytoskeleton during polarized morphogenesis. *Science*. 276:118–122.

Griffiths, D.J., N.C. Barbet, S. McCready, A.R. Lehmann, and A.M. Carr. 1995. Fission yeast *rad17*: a homologue of budding yeast RAD24 that shares regions of sequence similarity with DNA polymerase accessory proteins. *EMBO (Eur. Mol. Biol. Organ.) J*. 14:5812–5823.

Gutz, H., H. Heslot, U. Leupold, and N. Loprieno. 1974. *Schizosaccharomyces pombe*. *In* Handbook of Genetics. Vol. 1. R.C. King, editor. Plenum Publishing Corp., New York. 395–466.

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

Hagan, I., and M. Yanagida. 1995. The product of the spindle formation gene *sad1*[+] associates with the fission yeast spindle pole body and is essential for viability. *J. Cell Biol*. 129:1033–1047.

Hagan, I., and M. Yanagida. 1997. Evidence for cell cycle-specific, spindle pole body-mediated, nuclear positioning in the fission yeast *Schizosaccharomyces pombe*. *J. Cell Sci*. 110:1851–1866.

Hanahan, D. 1985. Techniques for transformation of *E. coli*. *In* DNA Cloning, a Practical Approach. D.M. Glover, editor. 109–135. IRL Press, New York.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

Harris, S.D., L. Hamer, K.E. Sharpless, and J.E. Hamer. 1997. The *Aspergillus nidulans SEPA* gene encodes an FH1/FH2 protein involved in cytokinesis and the maintenance of cellular polarity. *EMBO (Eur. Mol. Biol. Organ.) J*. 16:3474–3483.

Heim. R., A.B. Cubitt, and R.Y. Tsien. 1995. Improved green fluorescence. *Nature*. 373:663–664.

Ho, S.N., H.D. Hunt, R.M. Horton, J.K. Pullen, and L.R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene*. 77:51–59.

Imamura, H., K. Tanaka, T. Hihara, M. Umikawa, T. Kamei, K. Takahashi, T. Sasaki, and Y. Takai. 1997. Bni1p and Bnr1p: downstream targets of the Rho family small G-proteins which interact with profilin and regulate actin cytoskeleton in *Saccharomyces cerevisiae*. *EMBO (Eur. Mol Biol. Organ.) J*. 16:2745–2755.

Jansen, R.-P., C. Dowzer, C. Michaelis, M. Galova, and K. Nasmyth. 1996. Mother cell-specific *HO* expression in budding yeast depends on the unconventional myosin Myo4p and other cytoplasmic proteins. *Cell*. 84:687–697.

Jackson-Grusby, L., A. Kuo, and P. Leder. 1992. A variant *limb deformity* transcript expressed in the embryonic mouse limb defines a novel formin. *Genes & Dev*. 8:2123–2136.

Kocher, T.D., W.K. Thomas, A. Meyer, S.V. Edwards, S. Paabo, F.X. Villablanca, and A.C. Wilson. 1989. Dynamic of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA*. 86:6196–6200.

Kohno, H., K. Tanaka, A. Mino, M. Umikawa, H. Imamura, T. Fujiwara, Y. Fujita, K. Hotta, H. Qadota, T. Watanabe, et al. 1996. Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in *Saccharomyces cerevisiae*. *EMBO (Eur. Mol. Biol. Organ.) J*. 15:6060–6068.

Kilmartin, J.V., and A.E.M. Adams. 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomyces cerevisiae*. *J. Cell Biol*. 98:922–939.

Kjærulff, S., J. Davey, and O. Nielsen. 1994. Analysis of the structural genes encoding M-factor in the fission yeast *Schizosaccharomyces pombe*: identification of a third gene, *mfm3*. *Mol. Cell Biol*. 14:3895–3905.

Lupas, A., M. Van Dyke, and J. Stock. 1991. Predicting coiled coils from protein sequences. *Science*. 252:1162–1164.

Marhoul, J.F., and T.H. Adams. 1995. Identification of developmental regulatory genes in *Aspergillus nidulans* by overexpression. *Genetics*. 139:537–547.

Marks, J., and J.S. Hyams. 1985. Localization of F-actin through the cell division cycle of *Schizosaccharomyces pombe*. *Eur. J. Cell Biol*. 39:27–32.

Maundrell, K. 1993. Thiamine-repressible expression vectors pRep and pRip for fission yeast. *Gene*. 132:127–130.

Meloche, S., G. Pagés, and J. Pouyssegur. 1992. Functional expression and growth factor activation of an epitope-tagged p44 mitogen-activated protein kinase, p44[mapk]. *Mol. Biol. Cell*. 3:63–71.

Moreno, S., A. Klar, and P. Nurse. 1991. An introduction to molecular genetics analysis of the fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol*. 194:795–823.

Nielsen, O., and R. Egel. 1990. The *pat1* protein kinase controls transcription of the mating-type genes in fission yeast. *EMBO (Eur. Mol. Biol. Organ.) J*. 9:1401–1406.

Nielsen, O., J. Davey, and R. Egel. 1992. The *ras1* function of *Schizosaccharomyces pombe* mediates pheromone-induced transcription. *EMBO (Eur. Mol. Biol. Organ.) J*. 11:1391–1395.

Nielsen, O., and J. Davey. 1995. Pheromone communication in the fission yeast *Schizosaccharomyces pombe*. *Semin. Cell Biol*. 6:65–104.

Petersen, J., D. Weilguny, R. Egel, and O. Nielsen. 1995. Characterization of *fus1* of *Schizosaccharomyces pombe*: A developmentally controlled function needed for conjugation. *Mol. Cell. Biol*. 15:3697–3707.

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

Robinow, C.F., and J.S. Hyams. 1989. General cytology of fission yeast. *In* The Molecular Biology of the Fission Yeast. A. Nasin, P.G. Young, and B.F. Johnson, editors. 273–331. Academic Press, New York.

Roemer, T., L.G. Vallier, and M. Snyder. 1996. Selection of polarized growth sites in yeast. *Trends Cell Biol*. 6:434–441.

Rose, M., F. Winston, and P. Heiter. 1990. Methods in Yeast Genetics: A Laboratory Course Manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbour Laboratory Press. Cold Spring Harbor, NY.

Simon, M.-N., C.D. Virgilio, B. Souza, J.R. Pringle, A. Abo, and S.I. Reed. 1995. Role for the Rho-family GTPase Cdc42 in yeast mating-pheromone signal pathway. *Nature*. 376:702–705.

Sohn, R.H., and P.J. Goldschmidt-Clermont. 1994. Profilin: at the crossroads of signal transduction and the actin cytoskeleton *Bioessays*. 16(7):465–472.

Studier, F.W., A.H. Rosenburg, J.J. Dunn, and J.W. Dubenhoff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol*. 185:60–89.

Sudol, M. 1996. The WW module competes with the SH3 domain? *Trends Biochem. Sci*. 21:161–163.

Tanaka, M., and H. Shibata. 1985. Poly (ʟ-proline)-binding proteins from chick embryos are a profilin and a profilactin. *Eur. J. Biochem*. 151:291–297.

Vallen, E.A., W. Ho, M. Winey, and M.D. Rose. 1994. Genetic interactions between Cdc31 and Kar1, 2 genes required for duplication of the microtubule-organizing center in *Saccharomyces cerevisiae*. *Genetics*. 137:407–422.

Waddle, J.A., T.S. Karpova, R.H. Waterston, and J.A. Cooper. 1996. Movement of cortical actin patches in yeast. *J. Cell Biol*. 132:861–870.

Watanabe, N., P. Madaule, T. Ried, T. Ishizaki, G. Watanabe, A. Kakizuka, Y. Saito, K. Nakao, B.M. Jockusch, and S. Narumiya. 1997. p140mDia, a mammalian homolog af *Drosophila* diaphanous, is a target protein for rho small GTPase and is a ligand for profilin. *EMBO* (*Eur. Mol. Biol. Organ.*) *J*. 16: 3044–3056.

Weilguny, D., M. Prœtorius, A. Carr, R. Egel, and O. Nielsen. 1991. New vectors in fission yeast: application for cloning the *his2* gene. *Gene*. 99:47–54.

Wilson, I.A., H.L. Niman, R.A. Houghten, A.R. Cherenson, M.L. Connolly, and A.R. Lerner. 1984. The structure of an antigen determinant in a protein. *Cell*. 37: 767–778.

Woychik, R.P., R. Maas, R. Zeller, T. Vogt, and P. Leder. 1990. Formins: proteins deduced from alternative transcripts of the *limb deformity* gene. *Nature*. 346:850–852.

Zaitsevskaya-Carter, T., and J. Cooper. 1997. Spm1, a stress-activated MAP kinase that regulates morphogenesis in *S. pombe*. *EMBO (Eur. Mol. Biol. Organ.) J*. 16:1318–1331.