Arp10p Is a Pointed-End-associated Component of Yeast Dynactin[□]

Sean W. Clark and Mark D. Rose

Department of Molecular Biology, Princeton University, Princeton, NJ 08544

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In metazoans, dynein-dependent vesicle transport is mediated by dynactin, containing an actin-related protein, Arp1p, together with a cargo-selection complex containing a second actin-related protein, Arp11. Paradoxically, in budding yeast, models of dynactin function imply an interaction with membranes, whereas the lack of microtubule-based vesicle transport implies the absence of a cargo-selection complex. Using both genetic and biochemical approaches, we demonstrate that Arp10p is the functional yeast homologue of Arp11, suggesting the possible existence of a pointed-end complex in yeast. Specifically, Arp10p interacts with Arp1p and other dynactin subunits and is dependent on Arp1p for stability. Conversely, Arp10p stabilizes the dynactin complex by association with the Arp1p filament pointed end. Using a novel hRAS–Arp1p one-hybrid assay, we show that Arp1p associates with the plasma membrane dependent on dynactin subunits, but independent of dynein, and sensitive to cell wall damage. We directly show the association of Arp1p with not only the plasma membrane but also with a less dense membrane fraction. Based on the hRAS–Arp1p assay, loss of Arp10p enhances the apparent association of dynactin with the plasma membrane and suppresses the loss of signaling conferred by cell wall damage.

INTRODUCTION

Cytoplasmic dynein and its regulator dynactin have multiple roles in metazoans during interphase and mitosis. The dynactin complex assists cytoplasmic dynein by increasing its processivity and by regulation of cargo binding via the pointed-end complex (Eckley *et al.*, 1999; Karki *et al.*, 2000; King and Schroer, 2000). The importance of dynein's dynactin-dependent transport is emphasized by the recent discovery of a dynactin mutation that results in motor neuron degeneration (LaMonte *et al.*, 2002; Puls *et al.*, 2003). Although this mutation affects dynactin's interaction with microtubules, it follows that cargo-selection is likely important as well. Many of dynein's functions during mitosis have been gleaned from budding yeast; however, the apparent absence of a vesicular transport role in interphase has thus far limited its usefulness for understanding other facets of dynein function.

Searches for budding yeast representatives of the pointed-end complex have not been fruitful (Eckley *et al.*, 1999), consistent with the apparent absence of microtubule-dependent vesicle transport (Huffaker *et al.*, 1988). The metazoan dynactin complex contains two Arps: Arp1 and Arp11, consistent with the general pattern of paired Arps engaged in related functions (Arp2/Arp3 and Arp7/Arp9) (Goodson and Hawse, 2002). Arp1p forms a short filament that is the scaffold for the remaining dynactin subunits. Like actin, Arp1p filaments are polarized, with analogous barbed and

pointed ends. A cargo-selection complex, including Arp11, is bound to the pointed end of Arp1p in metazoans (Eckley *et al.*, 1999). A recent phylogenetic analysis of the actin family (Goodson and Hawse, 2002) and two-hybrid data suggested that Arp1p and Arp10p do interact (Uetz *et al.*, 2000), leading us to revisit the possibility that Arp10p is part of a pointed-end complex in yeast.

In addition to its role in mitosis, dynactin, but not dynein, is involved in a cell wall synthesis checkpoint (Suzuki *et al.*, 2004). On suffering cell wall damage, budding yeast cells arrest after DNA replication and spindle pole body (SPB) duplication, but before SPB separation and spindle assembly. Cell wall damage-induced arrest is dependent on *wac1*, an allele of *ARP1*. The checkpoint was dependent on two other dynactin subunits, Jnm1p and Nip100p, but not Arp10p (Suzuki *et al.*, 2004). The checkpoint can be induced by mutations in the redundant glucan synthases, *FKS1* and *FKS2*. Deletion of both *FKS1* and *FKS2* results in lethality. During normal vegetative growth, *FKS1* is the predominant transcript; however, *FKS2* is induced in the absence of *FKS1* or during stress conditions (Mazur *et al.*, 1995). Consistent with a role in responding to cell wall damage, *ARP1* shows a synthetic defect with mutation of *SMI1*, a glucan synthesis regulator whose absence leads to a highly permeable cell wall (Lesage *et al.*, 2004).

MATERIALS AND METHODS

Strains and Yeast Methods

General yeast methods, media, and plasmid transformation were as described previously (Rose *et al.*, 1990).

Deletion of *ARP10*

The *arp10Δ::kanMX* cassette (Wach *et al.*, 1994) was amplified using a mixture of Taq (Roche Diagnostics, Indianapolis, IN) and Vent (New England Biolabs, Beverly, MA) polymerases (5:1 by units) with primers P147 and P148 and pMR5246 as a template with the following program: 1 cycle of 94°C 2 min followed by 20 cycles of 94°C 30 s, 54°C 30 s, 72°C 90 s and completed with

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Address correspondence to: Mark D. Rose (mrose@molbio.princeton.edu).

10 min at 72°C. The cassette was integrated into a diploid strain MY8891 (a cross of MY8885 and MY8665), and the deletion was confirmed by PCR with P149–P152 and P132, P133. After sporulation and dissection, the tetrads were examined at 14, 23, 30, and 37°C. To examine possible synthetic lethal interactions, *arp10Δ::kanMX* haploids (MY8892 and MY8893) were in turn crossed to other deletions in *ARP1* (MY8663), *JNM1* (MY8887), *DYN1* (MY8671), *KIP2* (MS2405), and *KIP3* (MY8660) and subjected to tetrad analysis.

Analysis of *arp1* Alleles in the *arp10Δ* Background

MY8892 and MY8655 were crossed, sporulated, and dissected to produce MY8949 (*arp10Δ kip3-15*). MY8949 was in turn crossed to several *arp1* alleles, *ARP1*, and *arp1Δ*. Tetrads were examined on 5-fluoroorotic acid at 14, 23, 30, and 37°C.

Examination of Anaphase in *arp10Δ* Strains

Strains harboring *ARP1*, *arp1Δ*, or *arp10Δ* were grown in YPD at 11°C. A one-tenth volume of formaldehyde was added to the culture and incubated for 30 min at 11°C. Approximately 1×10^8 cells were collected and resuspended in 100 μl of water; 1 ml of methanol was added for 10 min at room temperature. The cells were pelleted and washed once in 1 ml of water and repelleted. Then, 50 μl of 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) was added for 5 min. Finally, the cells were washed three times with 1 ml of H₂O. Next, 50 μl of cells was mixed with 50 μl of 1% low-melting point agarose, and 2 μl was placed on a slide under a coverslip and examined by epifluorescent and differential interference contrast microscopy using a DeltaVision microscope (Applied Precision, Seattle, WA). Images covering a 0.25-mm² area were collected, and the fraction of cells with two DAPI spots in the mother cell was determined.

Construction of Plasmid for Overexpression of *Arp10p*

pMR5585 was created from the 2 μ plasmid pMR1869, cut with *Bam*HI and *Xho*I, and cotransformed into a His⁺ strain with a PCR product containing *ARP10* amplified from genomic DNA with P539 and P540. The gap-repaired plasmid was confirmed by sequencing.

Flotation of *Arp1p*

Membranes were isolated and separated by isopycnic centrifugation essentially as described in Roberg *et al.* (1997). Briefly, 4.1×10^8 cells were collected and washed in 20 ml of 5 mM Tris-Cl, pH 7.4. These cells were resuspended in 500 μl of STE10 + protease inhibitors [10% (wt/wt) sucrose, 10 mM Tris-Cl, pH 7.4, 10 mM EDTA, 1 mg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), and 5 μg/ml each chymostatin, pepstatin, antipain, arpotinin, and leupeptin]. The cell suspension was added to prechilled glass beads (0.5 mm; BioSpec Products, Bartlesville, OK), and the cells were lysed with a Bead-Beater (BioSpec Products) for 1.5 min at 4°C. Crude lysates were clarified twice at $300 \times g$ for 2 min in a microcentrifuge at 4°C. Membranes were isolated with a TLA100 rotor at 100,000 rpm for 10 min at 4°C. The membrane pellet was resuspended in 400 μl of 67% (wt/wt) sucrose, 10 mM Tris-Cl, pH 7.4, and 10 mM EDTA with a Dounce homogenizer. Then, 100 μl of resuspended membranes was layered under 12 ml of 20–60% (wt/wt) sucrose gradients in 10 mM Tris-Cl, pH 7.4, 10 mM EDTA. Gradients were placed in an SW-41 rotor (Beckman Coulter, Fullerton, CA) at 34,000 rpm for 23.3 h at 4°C. Gradients were fractionated (1 ml) from the top. Then, 30 μl of each fraction was analyzed by SDS-PAGE and immunoblotting. In some cases, gradient fractions were trichloroacetic acid (TCA) precipitated before analysis by SDS-PAGE. In this case, 3 μl of 2% NaDOC was added to 300 μl of each fraction and placed on ice for 30 min. Then, 600 μl of ice-cold 15% TCA was added, and the samples were kept on ice overnight. Precipitates were isolated in a microcentrifuge for 10 min at 4°C, washed with 500 μl of –20°C acetone, and reisolated. Antibodies against VPH1 (10D7), Porin (16G9), PGK (22C5), DPM1 (5C5), PEP12 (2C3), and VPS10 (18C8) were obtained from Invitrogen (Carlsbad, CA). The mouse anti-Ras antibody was obtained from BD Biosciences (San Jose, CA). The anti-PMA1 (40B7) antibody was obtained from Abcam (Cambridge, MA).

Rate-Zonal Sedimentation

Cytosols were prepared as described above, except the lysis buffer contained 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM ATP, 1 mM dithiothreitol (DTT), 0.5 mM Na₃VO₄, 10 mM NaF, and 10 mM Na₂SO₄ plus protease inhibitors as described above except 1 mg/ml AEBSF was substituted for phenylmethylsulfonyl fluoride (PMSF). Then, 500 μl of cytosol was loaded atop 11 ml of 5–20% (wt/vol) sucrose gradients made in lysis buffer without protease inhibitors. These were centrifuged in an SW-41 rotor (Beckman Coulter) at 4°C, 34,000 rpm, for 17 h. Then, 1-ml fractions were taken from the bottom. 10 μl of the load, and 30 liters of the fractions was mixed with 5× sample buffer and resolved by SDS-PAGE.

Tagging of *Arp1p*, *Arp10p*, *Jnm1p*, and *Nip100* with hemagglutinin (HA) and MYC

HA::kanMX and MYC::kanMX cassettes were amplified from templates pMR5093 and pMR5096, respectively, using a 5:1 mixture (by units) of Taq and Vent polymerases with the same program as the *arp10Δ::kanMX* cassette (Longtine *et al.*, 1998). The primers have 40–45 base pairs of homology to the 3' end of each open reading frame (ORF), such that the HA and MYC tags are added at the C terminus of each protein (P126, P127 [*ARP1*]; P128, P129 [*ARP10*]; P199, P200 [*JNM1*]; P162, P188 [*NIP100*]). The cassettes were integrated into MY8885 and confirmed by PCR (P31, P231 [*ARP1*]; P151, P152 [*ARP10*]; P213, P214 [*JNM1*]; P166, P167 [*NIP100*]) all in combination with P132 and P133). To create doubly tagged strains, *ARP1*–HA, *ARP1*–MYC, and *ARP10*–HA were first crossed to MY8665 to obtain tagged ORFs with the opposite mating type. These were crossed in turn to other tagged strains. Doubly marked haploids were chosen from kan^R segregants of NPD tetrads with respect to kan^R resistance (2:2).

Coimmunoprecipitation

Cytosols were prepared as described previously (Clark and Rose, 2005). Cytosol (50 μl) was centrifuged for 10 min at 4°C and then diluted into ice-cold buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% (wt/vol) NP-40, and 40 μg/ml PMSF]. Immune complexes were formed with 50 μl of a 1:1 slurry of anti-HA Affinity matrix beads (Roche Diagnostics). The beads were agitated for 2 h at 4°C and then washed three times in 1 ml of buffer, 5 min on ice. Protein was eluted from the beads with 2× SB. Samples were separated by SDS-PAGE and immunoblotted using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ) with 12CA5 (HA) or 9E10 (MYC) monoclonal antibody. Controls had only MYC-tagged proteins in an anti-HA immunoprecipitation (IP).

Protein Methods

Protein extracts were prepared from 50 ml of overnight cultures resuspended to 2.4×10^7 cells/ml in 50 ml of fresh media pre-equilibrated to the desired temperature. Cells were harvested after 3 h growth at the specified temperature. Cell pellets were resuspended at 1.2×10^9 cells/ml in freshly prepared ice-cold lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM Na₂ATP, 1 mM DTT, 40 μg/ml PMSF, and 5 μg/ml each chymostatin, pepstatin, antipain, aprotinin, and leupeptin). One milliliter of resuspended cells was added to prechilled glass beads (0.5 mm; BioSpec Products). Cells were lysed with a BeadBeater (BioSpec Products) for 1.5 min at 4°C. Crude lysates were clarified in a microcentrifuge for 5 min at 4°C. For cytosols, crude extracts were centrifuged 10 min (4°C) in a TLA rotor at 50,000 rpm. Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as a control. For analysis of steady-state protein levels, an equal quantity of total protein was added to each well, proteins were resolved by SDS-PAGE, and the relative quantity of *Arp1p* was determined by immunoblotting with an *Arp1p* antibody (Clark and Rose, 2005).

Rate-Zonal Sedimentation

Cytosols were prepared as described above, except the lysis buffer contained 20 mM Bis-Tris-Cl, pH 6.25, 1 mM ATP, and 2 mM DTT plus protease inhibitors as mentioned above except 1 mg/ml AEBSF was substituted for PMSF. Then, 500 μl of cytosol was loaded atop 11 ml of 5–20% sucrose gradients made in lysis buffer without protease inhibitors. These were centrifuged in an SW-41 rotor (Beckman Coulter) at 4°C, 34,000 rpm, for 17 h. One-milliliter fractions were taken from the bottom. Then, 10 μl of the load and 30 liters of the fractions were mixed with 5× sample buffer and resolved by SDS-PAGE.

Two-Hybrid Interactions

ARP10 was amplified using Pwo polymerase (Roche Diagnostics) from MY8648 genomic DNA with primers P258 and P259, which include *Bam*HI sites, with the following program (1 cycle at 94°C 2 min followed by 30 cycles of 94°C 30 s, 58°C 30 s, 72°C 30 s, and a final extension at 72°C 10 min). The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of pMR1869, producing pMR5391. *ARP10* was confirmed by sequencing. *ARP1* two-hybrid bait and prey plasmids were prepared in both pMR5353 (pGBDU-C1) and pMR5389 (pGAD-C1) by linearization with *Eco*RI followed by fill-in with Klenow DNA polymerase. An *Nco*I–*Xba*I fragment from pMR5388 (Clark and Rose, 2005) was filled in with Klenow DNA polymerase and ligated to the linearized vector. The correct orientation was confirmed by digestion with *Clal*. The pMR5370 bait was used to screen a library, amplified as described previously (James *et al.*, 1996), by the TRAF0 method (Gietz and Schiestl, 1995) of pGAD-C1 prey plasmids that activated the *ADE2* reporter by selection on SC-Leu-Ura-Ade. Sequencing of one such prey plasmid (pMR4212) confirmed that it contained an in-frame fusion to *JNM1* at the third codon. To construct *ARP10* bait and prey plasmids, primers containing sequence homologous to either pMR5353 (pGBDU-C1, P264/P265) or pMR3761 (pGAD-C1, P266/P267) were used to amplify *ARP10* from the pMR5391 template. A (5:1) mixture of Taq and Vent polymerases was used with the following

program: 1 cycle at 94°C 2 min followed by 20 cycles of 94°C 30 s, 55°C 30 s, 72°C 30 s, and a final extension at 72°C 10 min. For *ARP1* alleles, plasmids harboring wild-type *ARP1* or *ARP1* alleles (Clark and Rose, 2005) were used as templates with primers P193/P194 (pMR5353) or P262/P263 (pMR3761). Insertion of the target PCR product into pMR3761 and pMR5353 plasmids was achieved by cotransformation of MY8648 with the appropriate PCR product and linearized two-hybrid plasmid. pMR3761 was digested with *Bam*HI and *Sal*I; pMR5353 was digested with *Bam*HI and *Eco*RI. After selection of transformants on synthetic media lacking uracil or leucine, plasmids were extracted and transformed into *Escherichia coli* strain XL1-Blue. Plasmid DNA was prepared and examined for proper insertion of the target sequence by restriction digest. Finally, plasmids were transformed into the appropriate two-hybrid tester strain.

Doxycycline Repression of *ARP1* in *arp10Δ*

arp10Δ::HPH was integrated into a strain harboring *ARP1* under control of a tetracycline-repressible promoter in a *kipp3Δ* background (MY8662), producing MY8974. Solid media containing doxycycline was prepared by addition from a 5 mg/ml stock in 50% ethanol as 1:3 dilutions ranging from 10 to 0.014 μg/ml.

RAS-*ARP1* In Vivo Membrane Association Assay

We used a system (Broder *et al.*, 1998) wherein a human activated allele of RAS, under control of a methionine-repressible promoter, lacks sequences that normally direct it to the plasma membrane. This construct is tested in a strain harboring a conditional allele of the exchange factor CDC25. At the restrictive temperature, endogenous RAS is inactive because of the *cdc25-2* mutation. This strain can be rescued by protein fusions that redirect activated human RAS to the membrane. To create pMR5422, *ARP1* was amplified from pMR5352 with P334, P335 and as a C-terminal fusion of RAS in pMR5421 linearized with *Eco*RI/*Xho*I, by gap repair. RAS-*ARP1* was moved via *Hin*dIII/*Xho*I to pMR5420, creating pMR5423. In parallel, RAS was moved from pMR5421 to produce pMR5424. The resulting constructs were sequenced then transformed into the tester strain MY9035. The tester strain was created by inserting a resistance marker for hygromycin, amplified with P355, P356 from pMR5390 (Goldstein and McCusker, 1999), downstream of the *cdc25-2* mutation in MY8304 to create MY9035. MY9035 was backcrossed to MY8665 for MATa, creating MY9036. MY9036 was in turn crossed to strains harboring deletions in *DYN1* (MY8890), *NUM1* (MY9008), *JNM1* (MY8646), *NIP100* (MY8669), or *ARP10* (MY8894). Strains MY9589, MY9590, and MY9591 were derived from crossing MY9586 × MY9035. MY9586 was derived from a cross between MY8894 and MY9581 (a gift from Y. Ohya, University of Tokyo, Tokyo, Japan; YOC1087). Plasmid MR5527 was created by amplifying RAS and a primer-borne CAAX sequence from MR5421 with P486/P487 and cotransforming with *Hin*dIII/*Xho*I-digested MR5420 for assembly by gap repair. Plasmid MR5528 was created by amplifying RAS with P482/P483 from MR5421 and assembly by gap repair into MR5120 digested with *Eco*RI/*Xho*I. MR5528 then served as template for P484/P485 for gap repair into MR5420 digested with *Hin*dIII/*Xho*I, to create MR5529. The RAS, RAS-*ARP1*, Myristoyl-RAS, and RAS-CAAX fusions, all under control of the *MET25* promoter, were induced by growth on media lacking methionine and leucine at the indicated temperature.

Alignment of *ARP1* and *ARP10* and Modeling of *Arp10p* on the *Arp1p* Filament Model

Because of its low sequence similarity with actin, *Arp10p* cannot be modeled by homology-based methods; hence, we approximated the position of absent residues on the *Arp1p* model (Clark and Rose, 2005). Residues of *Arp1p* absent in *Arp10p* were determined based on an alignment of *ARPs* (Poch and Winsor, 1997).

RESULTS

Arp10 and *Arp11* Are Both Predicted to Lack Subdomains Required for Pointed-End Polymerization

Metazoan *Arp11* and budding yeast *Arp10p* are conspicuous among the *Arps* for their small size, owing to large in-frame deletions relative to the other *Arps*. Like *Arp11* (Eckley *et al.*, 1999), when *Arp10p* is mapped onto the predicted structure of *Arp1p* modeled into a filament, the deletions are not random; most are found at *Arp10p*'s pointed end. Small insertions would occur near the predicted interface between *Arp1p* and *Arp10p* (Figure 1A). This is consistent with a pointed-end capping protein, i.e., one that can add on to the *Arp1p* filament but cannot support further elongation.

Figure 1. Structural and genetic evidence for *Arp10p* interaction with the *Arp1p* filament. (A) *Arp10p* modeled at the pointed end of *Arp1p* (gray). *Arp10p* (blue) shows the position of deleted residues (red) or small insertions (yellow). Deletions are at the pointed end, where they would prevent association of further subunits; insertions are near the heterotypic interface where they could exert stabilizing forces. *ARP1* alleles used in this study are indicated in green. (B) Genetic interaction of *arp10Δ* with *ARP1*. Growth was examined with *kipp3-15* which renders *ARP1* essential, although in this strain background *kipp3-15 arp1Δ* or *kipp3Δarp1Δ* provide for weak growth at 23°C. *kipp3-15* and *kipp3Δ* are equivalent under the conditions examined (Clark and Rose, 2005). Deletion of *ARP10* exacerbates both and *Ts⁻* (*arp1-113*) and specific pseudo wild-type alleles (*arp1-44*, *arp1-46*), but not others (*arp1-37*). (C) Deletion of *ARP10* increases wild-type *ARP1* protein threshold for growth. The *ARP1* ORF is driven by a doxycycline-repressible promoter in a *kipp3Δ* TET-SSN6 background. Addition of doxycycline (10 μg/ml) decreases *Arp1p* levels below the threshold for wild-type growth. Removal of *Arp10p* further enhances this defect. The unexpressed promoter has slightly lower levels of *Arp1p*, evident as a reduction of growth in the absence of *Arp10p*. 1:10 serial dilutions are shown. (D) Deletion of *ARP10* abates overexpression rescue of specific *ARP1* mutants in *kipp3-15*. Overexpression rescues particular *Ts⁻* and lethal *ARP1* mutants. Elimination of *Arp10p* inhibits remediation of specific alleles.

ARP10 Exhibits Genetic Interactions with Dynactin

A simple genetic test has been used to assign proteins to the dynactin/dynein pathway in budding yeast. In yeast, the mitotic spindle forms in the mother cell and then must move into the bud. Two sequential pathways align and move the mitotic spindle into the bud. The first is dependent on the kinesin *KIP3*; the second is dependent on dynactin/dynein. Either pathway is sufficient to complete the task, albeit with lower efficiency; however, the absence of both results in "synthetic lethality" (Cottingham and Hoyt, 1997; DeZwaan *et al.*, 1997). To date, all known dynactin subunits are not essential for life, but their deletions result in synthetic lethality with *KIP3* mutations. We found *ARP10* was also not essential (Figure 1B); however, Arp10p did not show synthetic lethality with either *kip3Δ*, any of the dynactin/dynein subunits *arp1Δ*, *jnm1Δ*, *dyn1Δ*, or with the kinesin *kip2Δ*.

A second test of proteins acting in the dynein pathway concerns the timing and position of spindle elongation. Normally, anaphase initiates at the same time as insertion of the spindle through the bud neck and the occurrence of anaphase entirely within the mother cell is a hallmark of dynein mutants. Whereas *arp1Δ* cells exhibited 10.4% anaphase in the mother cells, *arp10Δ* cells exhibited 2.2% anaphase in the mother cells, similar to the wild-type value of 1.1%. These observations are consistent with the reported absence of a spindle partitioning defect for *arp10Δ* (Eckley and Schroer, 2003).

These results certainly indicate that Arp10p does not play a critical role in spindle orientation. However, they do not challenge the possibility that Arp10p interacts with the dynactin complex or that Arp10p is involved in other functions of the dynactin complex. We reasoned that if Arp10p is a dynactin subunit, then loss of Arp10p might cause a detectable phenotype in a *kip3Δ* background, if overall dynactin function or stability were otherwise compromised. Accordingly, we used two methods to reduce dynactin activity: 1) conditional *ARP1* alleles and 2) reduced wild-type dynactin levels. First, using a comprehensive collection of *ARP1* alanine-scanning alleles (Table S1; Clark and Rose, 2005), we found that the temperature-sensitive phenotypes of several mutants were exacerbated in the absence of Arp10p. Several temperature-sensitive (*Ts*⁻) alleles even became nonconditional lethal mutations (e.g., *arp1-35*, *arp1-113*) (Figure 1B). Second, because some conditional *ARP1* alleles exhibit reduced steady-state levels of Arp1p, we considered that Arp10p might become critical as Arp1p protein levels became limiting. Budding yeast maintains about a 10-fold excess of Arp1p over that necessary for normal growth (Clark and Rose, 2005). We therefore determined whether the absence of Arp10p altered the minimum threshold at which Arp1p is required for growth. Arp1p levels were controlled by placing it under a doxycycline-repressible promoter (Belli *et al.*, 1998a,b). This produces slightly lower levels of Arp1p than its natural promoter (Clark and Rose, 2005). In the absence of Arp10p, the reduced levels of Arp1p caused reduced growth rates at both high and low temperatures, which were further diminished when Arp1p levels were repressed by addition of doxycycline (Figure 1C). However, this growth defect was less severe than when *arp10Δ* was combined with *Ts*⁻ alleles whose steady-state Arp1p protein levels are above the minimum threshold (Figure 1B). These results suggest that reduced Arp1p protein levels as well as reduced Arp1p function, result in the growth defect of the *Ts*⁻ mutants.

Because lowered Arp1p protein levels made cells sensitive to loss of Arp10p, we also examined *ARP1* pseudo wild-type

alleles, which generally exhibited wild-type protein levels. By definition, these alleles show no synthetic interactions with the *KIP3* pathway. Nonetheless, certain pseudo wild-type alleles became *Ts*⁻ in the absence of Arp10p (e.g., *arp1-44* and *arp1-46*) (Figure 1B). Thus, although mutations in *ARP10* do not normally show synthetic lethality with the *KIP3* pathway, even subtle debilitation of the dynactin complex provided a means to observe the contribution of *ARP10*.

Figure 2. Two-hybrid interactions define Arp10p association with the dynactin complex. (A) Individual dynactin subunits were fused to the galactose activating domain (GAD) or DNA binding domain (GBD). Interaction was monitored with a *HIS3* reporter at 30°C. A 10-fold serial dilution is shown for each strain. The GBD-ARP10-GAD-JNM1 interaction was only apparent when endogenous *JNM1* was deleted. (B) Summary of dynactin complex interactions detectable by two-hybrid. Only Arp10p fails to interact with itself, consistent with a capping protein. In one configuration, endogenous Jnm1p interferes with the Arp10p-Jnm1p interaction. AD, GAL activation domain; BD, GAL DNA binding domain. The budding yeast dynactin complex is shown below with the pointed end to the right, adapted from Schafer *et al.* (1994). (C) Deletion of *ARP10* reduces the interaction of Arp1p-Arp1p, Jnm1p-Jnm1p, and Arp1p-Jnm1p. (D) Pseudo wild-type allele *arp1-46* reduces the interaction of Arp10p and Jnm1p with Arp1p, without decreasing the interaction of Arp1p-Arp1p. Note that the more distantly positioned *arp1-44*, which overlaps with *arp1-46*, does not weaken this interaction. (E) Pseudo wild-type alleles do not generally reduce protein interactions: *arp1-112* reduces the Jnm1p-Arp1p interaction, but not the interaction of Arp10p or Arp1p with Arp1p. HIS, *HIS3* reporter.

Figure 3. Physical interactions of Arp10p. (A) Arp10p protein can be coimmunoprecipitated (IP) with dynactin subunits. IP targets were tagged with HA, whereas the associated protein was tagged with MYC epitope. IPs with anti-HA antibody were detected by immunoblotting with anti-MYC antibody. Control IPs are shown on the right, prepared from strains lacking HA-tagged subunits, but containing MYC-tagged subunits. The molecular weight standards correspond to protein migration on the control blot only. (B) Association of Arp1p and Jnm1p is reduced without Arp10p. Jnm1p was coIP with Arp1p in the presence or absence of Arp10p as in A. Duplicates are shown. Arp1p and Jnm1p are stable without Arp10p (inset). (C) Sedimentation of Arp10-MYCp in the presence and absence of Arp1p, Jnm1p, or both proteins. Cytosols from Arp10-MYCp with or without Arp1p or Jnm1p were examined by rate-zonal sedimentation on 5–20% sucrose gradients. Fractions containing Arp10p were determined with an anti-MYC antibody. Fraction 1 is the bottom of the gradient. Equal volumes of each fraction were loaded in each lane. The lower panel represents the distribution of Arp10p as a fraction of the maximal Arp10p signal. (D) Arp10p is specifically unstable without Arp1p. Arp1p is stable in the absence of Arp10p and other dynein/dynactin subunits. Dynactin

Most *ARP1* Ts⁻ alleles (and certain lethal alleles) can be suppressed by overexpression of the mutant Arp1p protein from a 2 μ plasmid via mass action (Clark and Rose, 2005). This likely reflects sufficient restoration of protein interaction to produce a functional dynactin complex. If Arp10p participates in Arp1p function, then its absence could interfere with the overexpression suppression of *ARP1* mutants. Not only did loss of Arp10p hamper overexpression suppression but also it did so in an allele-specific manner (Figure 1D, compare *arp1-104* and *arp1-115* with *arp1-38* and *arp1-39*). This result is consistent with the hypothesis that different *ARP1* Ts⁻ mutants interfere with different protein interactions, including that of Arp10p with the dynactin complex. Importantly, one of these mutations was *arp1-104*, a lethal allele at the pointed end of the Arp1p filament whose effect on filament length suggested the existence of additional proteins at the pointed end of the filament (Clark and Rose, 2005). The second allele-specific interaction is *arp1-115*, an allele found to be important for Jnm1p binding to Arp1p (Clark and Rose, 2005). The role of Arp10p in this protein interaction would be consistent with a ternary interaction between Arp1p, Jnm1p, and Arp10p.

Arp10p Is Physically Associated with the Dynactin Complex

Given the genetic evidence for the interaction of *ARP10* with dynactin, we sought evidence of a physical association by two-hybrid interaction. Consistent with Arp10p associating with dynactin, positive two-hybrid interactions were observed among all configurations of Arp1p, Jnm1p, and Arp10p, with only two exceptions (Figure 2A). First, Arp10p did not interact with itself, as would be expected for a capping protein. Second, although Arp1p fused to the activation domain interacted with Jnm1p fused to the DNA binding domain, the reverse configuration (BD-Arp10p and AD-Jnm1p) only showed an interaction when endogenous Jnm1p was deleted (Figure 2B). One simple interpretation of this result is that endogenous Jnm1p competes for binding to Arp10p.

We next determined whether loss of Arp10p affected any of the two-hybrid interactions. Indeed, Arp1p-Arp1p, Arp1p-Jnm1p, and Jnm1p-Jnm1p all exhibited reduced associations in the absence of Arp10p, which was particularly evident at 30°C, suggesting Arp10p helps to stabilize these interactions (Figure 2C). Finally, we tested a spectrum of *ARP1* alleles to determine whether any affected the two-hybrid interaction. Most *ARP1* alleles led to reduced interaction between Arp1p and Arp10p. Remarkably, this included pseudo wild-type *ARP1* alleles that otherwise did not seem to affect function (e.g., *arp1-46*). *arp1-46* reduced Arp1p-Arp10p and Arp1p-Jnm1p interaction, without af-

fecting Arp1p-Arp1p (Figure 2D). In contrast, a second pseudo wild-type mutation (*arp1-112*) specifically eliminated the Arp1p-Jnm1p interaction without affecting Arp1p-Arp10p (Figure 2E). These data suggest that Arp10p interacts with Arp1p independently of Jnm1p. The fact that *arp1-112* is pseudo wild type and yet affects an interaction essential to dynactin function indicates that the two-hybrid assay is sensitive to subtle changes in protein interactions.

To confirm the physical interactions of Arp10p implied by the two-hybrid data, we examined coimmunoprecipitates of Arp10p with Arp1p, Jnm1p, and another dynactin subunit, Nip100p. Arp10p can be reciprocally coimmunoprecipitated with Arp1p and can immunoprecipitate Jnm1p and Nip100p as well (Figure 3A). Interestingly, the absence of Arp10p in the *arp10 Δ* strain weakened the association of Arp1p and Jnm1p in the immunoprecipitates (Figure 3B), and *jnm1 Δ* similarly weakened the interaction of Arp1p with Arp10p (our unpublished data). In addition, we examined the sedimentation behavior of MYC-tagged Arp10p on sucrose gradients in the presence and absence of Arp1p or Jnm1p (Figure 3C). Arp10p shows two sedimentation peaks. The slower sedimenting form we attribute to partial dissociation from the complex under the sedimentation conditions, whereas the faster sedimenting form likely represents an intact complex. Without Arp1p, the sedimentation of Arp10p is entirely the slow sedimenting form. In the absence of Jnm1p, Arp10p is also more slowly sedimenting, but in an apparently larger complex than the absence of Arp1p. One interpretation is that Arp10p can interact with Arp1p and Jnm1p independently. This model suggests the absence of Arp1p or Jnm1p leaves Arp10p associated with the remaining subunit. This is borne out by the further reduction in Arp10p sedimentation in the absence of both Jnm1p and Arp1p.

The stability of interacting proteins is often observed to be affected by the presence of their binding partners. We found that the level of Arp10p was specifically dependent on the presence of Arp1p (Figure 3D). This observation makes it highly improbable that Arp10p operates independently of Arp1p. Given this observation, it is likely that any mutation that dislodges Arp10p from Arp1p will result in decreased Arp10p levels. We tested several Ts⁻ (e.g., *arp1-35*) and pseudo wild-type *ARP1* alleles (e.g., *arp1-37*, *-44*, and *-46*) and found reduced levels of Arp10p in those mutants in which the two-hybrid and genetic data had previously predicted a weakened interaction (Figure 3E).

Arp10p Maps to the Pointed End of the Arp1p Filament

In metazoan dynactin, Arp11 interacts with the pointed end of the Arp1 filament (Eckley *et al.*, 1999). The placement of Arp10p at the pointed end of the Arp1p filament is supported by the genetic and biochemical properties of Arp10p. In particular, two overlapping pseudo wild-type mutations, *arp1-44* and *arp1-46*, mapping at the pointed end, showed a distinct pattern of genetic and physical interactions with Arp10p. The *arp1-44* and *arp1-46* alleles are different alanine-scanning mutations of an overlapping charge cluster with two residues in common and one residue unique. The overlapping residues and the unique lysine of the stronger allele (*arp1-46*) are positioned near two predicted Arp1p-Arp10p interfaces at the pointed end, whereas those of the weaker *arp1-44* allele are more distant from Arp10p (Figure 3F).

As a complementary approach, we examined the two-hybrid interactions between Arp10p and Arp1p's containing all of the mutations that map to the opposite, "barbed" end of the filament (Figure 4A, b). All of the barbed-end *ARP1* mutations were pseudo wild type, yet four alleles (*arp1-86*,

Figure 3 (cont.) subunits Jnm1p and Nip100p are stable without Arp1p, but Arp10p is uniquely unstable without Arp1p. (E) An in vivo assay for Arp10p association with Arp1p. The instability of Arp10p without Arp1p provides a readout of Arp10p association. The steady-state level of Arp10p protein is specifically reduced in those *ARP1* mutants showing genetic interaction. (F) Arp10p interaction with *ARP1* pseudo wild-type alleles on the Arp1p filament model. The unique residue of *arp1-46* and the two residues in common with *arp1-44* are positioned such that they could be important for Arp10p contact with two different Arp1p subunits. Note that *arp1-44*, the weaker of the two alleles in many assays for Arp10p interaction, is more distantly positioned from Arp10p. Arp10p is shown in blue; side chains of Arp10p, absent relative to Arp1p, are not shown. Two underlying Arp1p subunits are shown by wireframe.

Figure 4. Arp10p interaction with ARP1 pseudo wild-type alleles on the barbed end of Arp1p and rescue of ARP1 alleles by overexpression of Arp10p. (A) Two-hybrid results for both ARP10 and ARP1 interaction with ARP1 barbed-end pseudo wild-type alleles. No ARP1 allele reduces Arp10p–Arp1p interaction, consistent with the association of Arp10p at the pointed, rather than barbed, end of Arp1p. Note that some alleles (*arp1-86*, *-99*, *-176*, and *-181*) do reduce the Arp1–Arp1 homotypic interaction, demonstrating the allele specificity of the assay. (B) A model of the barbed end of an Arp1p pentamer indicating the position of all barbed-end alanine-scanning alleles, all of which are pseudo wild type. The alleles on only one monomer are labeled owing to the symmetry. (C) Rescue of ARP1 alleles by overexpression of Arp10p from a high-copy plasmid (2μ) was tested in 21 ARP1 alleles, ARP1, and *arp1Δ* at five temperatures. Only alleles demonstrating rescue are shown.

-99, *-176*, and *-181*) showed a reduced homotypic Arp1p–Arp1p interaction. Regardless, none of the barbed-end ARP1 alleles reduced the interaction between Arp1p and Arp10p, demonstrating the specificity of the pointed-end mutation *arp1-46*, in the two-hybrid assay.

We have previously shown the utility of mapping protein interactions through rescue of mutant alleles by overexpression of the binding partners (Clark and Rose, 2005). We overexpressed Arp10p from a high-copy plasmid in a representative group of 21 ARP1 mutants (Figure 4C). Seven of these Arp1p mutants were at least partly suppressed by overexpression of Arp10p, with less severe Arp1p mutations showing greater rescue. The mutations fell into three groups: those at the pointed end (*arp1-104*), those at the core of the Arp1p filament (*arp1-35*), and those along the Arp1p filament (*arp1-38*, *-39*, *-45*, *-100*, and *-115*). Given their locations, most of these alleles are likely to be rescued by stabilizing the Arp1p filament, although the pointed-end mutation may be rescued by a more direct mechanism. Regardless of mechanism, the rescue by Arp10p overexpression is indicative of enhanced interaction with the dynactin complex.

Arp1p Membrane Interaction Is Dynein Independent, Dynactin Dependent, and Reduced in the Presence of *Arp10p*

Dynein and dynactin are thought to provide the necessary force on the mitotic spindle by interaction with the cortex (Heil-Chapdelaine *et al.*, 2000). We explored the possibility that Arp1p might associate with membranes using an in vivo membrane interaction assay (Figure 5). This assay made use of an activated allele of human hRAS that cannot interact with membranes. The inclusion of a Ts⁻ mutation in the exchange factor, CDC25, responsible for the activation of endogenous Ras means the strain cannot grow at temperatures above 23°C (Broder *et al.*, 1998). Addition of signals for lipid modification or fusion of the mutant hRAS to a protein associated with the plasma membrane restores signaling

activity and viability at the restrictive temperatures of 35°C and above. Fusion of hRAS to Arp1p clearly restored growth (Figure 5A). The growth of hRAS–ARP1 was on par with that of two hRAS fusions that restore association of RAS with the plasma membrane: an N-terminal myristoylation signal (Myr–RAS) or restoration of the C-terminal CAAX residues to hRAS (RAS–CAAX). The hRAS–ARP1 fusion protein was still functional for Arp1p activity because it rescued the synthetic lethality between *arp1Δ* and *kip3Δ* (our unpublished data), and therefore can associate with other required components of the dynactin complex. We undertook a candidate-based search to probe the requirements for the Arp1p–membrane interaction. Mutations in dynein or dynein's cortical receptor Num1p (Farkasovsky and Kuntzel, 2001) had no effect. In contrast, deletion of Jnm1p, and to a lesser extent Nip100p, strongly inhibited growth, implying that these dynactin subunits are required for Arp1p's membrane interaction. Together, these results imply that the dynactin complex can interact with the plasma membrane independent of dynein and that Arp1p cannot become associated with the plasma membrane in the absence of crucial dynactin subunits. However, unlike Jnm1p and Nip100p, loss of Arp10p actually enhanced hRAS–Arp1p-dependent growth. This result was most evident at 37°C where *cdc25-2* activity is lowest. The increase in Ras activity in the absence of Arp10p has two interpretations. First, Arp10p may sterically interfere with Ras signaling. Second, Arp10p may sterically interfere with membrane association. If it is membrane association, then this observation suggests that the normal role of Arp10p is to negatively affect the association of Arp1p with the plasma membrane.

Results from the RAS–Arp1p assay implied an association between Arp1p and the plasma membrane. To address this directly, we examined the floatation of membranes in isopycnic gradients (Figure 5B). Although a considerable association between Arp1p and membranes was evident, only a small fraction of Arp1p was apparent at the density of the plasma membrane. Conversely, most Arp1p cofractionated

Figure 5. Arp1p interaction with the membrane is independent of dynein and is negatively affected by Arp10p. (A) An in vivo Ras-based one-hybrid assay reveals an association between Arp1p and the membrane. A *cdc25-2::HPH* T_s^- allele in the background of all strains shown eliminates the endogenous Ras activity at 35°C and above. Ras activity can be substituted by a plasmid harboring a heterologous activated Ras allele (hRAS*) that does not require the nucleotide exchange activity of CDC25. Because hRAS* lacks residues that would normally direct it to the plasma membrane, it cannot restore Ras activity. Addition of either an N-terminal myristoylation signal or restoration of the C-terminal CAAX sequence to hRAS* redirects hRAS* to the plasma membrane, overcoming the loss of endogenous Ras activity afforded by *cdc25-2*, and restoring growth. Fusion of hRAS* to Arp1p is equally capable of restoring activity. This interaction is not dependent on dynein or its receptor Num1p but is dependent on dynactin subunits Jnm1p and Nip100p. The elimination of Arp10p enhances the interaction, especially evident at 37°C. (B) Arp1p floats in buoyant density gradients with membranes. Isolated membranes were layered under a buoyant density gradient and allowed to reach equilibrium. Twelve 1-ml fractions were taken from the top of the gradient (left in figure) and a sample of each was resolved by SDS-PAGE. A portion of Arp1p floats to the same density as the plasma membrane; however, the majority of Arp1p is found associated with lighter membrane fractions. The position of various membranes was determined by immunoblotting for resident polypeptides. PGK is cytosolic, Pma1p is plasma membrane, Dpm1p is endoplasmic reticulum (ER) membrane, Pep12p is endosomal membrane, and Porin is mitochondrial outer membrane. The ER shows as a low-density membrane fraction due to the inclusion of EDTA.

with much lighter membranes. The identity of the lighter Arp1p-associated membranes awaits further analysis.

RAS-Arp1p Activity Is Reduced upon Perturbation of the Cell Wall and Restored by Elimination of Arp10p

The cell wall synthesis checkpoint, activated by perturbation of the cell wall, requires Arp1p and the dynactin subunits Jnm1p and Nip100p, but it is intact in the absence of Arp10p and the dynein pathway components Dyn1p and Num1p (Suzuki *et al.*, 2004). Elimination of *FKS1* activity is sufficient to induce the checkpoint, whereas *FKS2* has not been directly examined. To probe the involvement of Arp1p's association with the plasma membrane in the cell wall checkpoint, we examined the ability of Arp1p to direct hRAS to the plasma membrane in the absence of *FKS1* or *FKS2* activity (Figure 6A). The myristoylated hRAS and RAS-CAAX controls were not affected by the cell wall perturbation. Therefore, the RAS signaling pathway was not disturbed by the changes to the cell wall, and any inherent temperature-sensitive growth defects of the *FKS* mutants were not evident under these conditions. In contrast, when hRAS was

Figure 6. hRAS-Arp1p, but not RAS-CAAX signaling, is modulated by *FKS* and *ARP10*. (A) Introduction of *fks1Δ fks1-1154* or *fks2Δ* into the system does not perturb the activity of myristoylated hRAS* or hRAS*-CAAX; however, it eliminates the activity of hRAS*-ARP1. (B) The further elimination of *ARP10* (*arp10Δ*) remedies the perturbation of hRAS*-ARP1 caused by *fks1Δ fks1-1154*, *fks2Δ*, and *fks1Δ* alone.

fused to Arp1p, both the *fks1Δ fks1-1154* and the *fks2Δ* mutants exhibited severely reduced growth consistent with a reduction in Arp1p association with the plasma membrane or sequestration away from sites where hRAS can signal. Remarkably, hRAS-Arp1p-dependent growth was fully restored in both the *fks1Δ* and *fks2Δ* strains by elimination of Arp10p (Figure 6B). Together, these results suggest that deletion of Arp10p may affect the partitioning of hRAS-Arp1p to the plasma membrane.

DISCUSSION

The Absence of ARP10 KIP3 Synthetic Lethality

Mutations in all dynein and dynactin subunits show synthetic lethality with the *kip3* mutation because of the redundant mitotic roles of the dynein/dynactin and *KIP3* pathways. Considerable evidence indicates that Arp10p is a dynactin complex subunit. Why then do mutations in *ARP10* and *KIP3* fail to show synthetic lethality? We consider three possible explanations for the lack of synthetic lethality. First, dynactin may have roles other than spindle orientation in which Arp10p is more important. Second, a possible Arp1p filament-stabilizing role for Arp10p may only become important when Arp1p concentration is low or Arp1p's protein interactions are weakened. Finally, Arp10p's effects on dynactin association with the plasma membrane may not have a strong impact on dynactin's essential role in spindle orientation.

An Arp10p-Arp1p-Jnm1p Ternary Complex

There are five indications that a ternary complex is formed between Arp1p, Jnm1p, and Arp10p. First, all pairwise interactions were observed between these three proteins in the two-hybrid analysis. Interestingly, although GAD-Jnm1p and GBD-Arp10p interacted in wild-type cells, endogenous Jnm1p inhibited the reciprocal interaction between GBD-Jnm1p and GAD-Arp10p. This result might be observed if the intact native Jnm1p binds more strongly to GAD-Arp10p than does GBD-Jnm1p. These interactions could

either be direct or via bridging by the endogenous dynactin complex.

Second, we identified an *ARP1* allele (*arp1-46*), located at the pointed end of the filament, that reduced interactions with both Arp10p and Jnm1p, despite the fact that Jnm1p is not a pointed-end subunit. A second allele, exposed on the filament surface away from the pointed end (*arp1-112*), reduced the Arp1p–Jnm1p interaction but not the Arp1p–Arp10p interaction. One interpretation of the effect of the pointed-end allele would be that Arp10p contributes to the stability of the Arp1p–Jnm1p interaction, possibly as part of a ternary complex. In contrast, the effect of *arp1-112* demonstrates that Arp10p interacts with Arp1p independent of Jnm1p.

Third, immunoprecipitation experiments indicated that the interactions between Arp1p–Jnm1p and Arp1p–Arp10p were reduced in the absence of Arp10p and Jnm1p, respectively. These results lend further support to the hypothesis that a ternary interaction exists between Arp1p, Jnm1p, and Arp10p. Arp10p could stabilize the Arp1p–Jnm1p interaction by direct interaction with Jnm1p or indirectly through stabilizing the Arp1p filament.

Fourth, the sedimentation behavior of Arp10p in the absence of Jnm1p and Arp1p suggests that Arp10p is capable of independently interacting with these two proteins.

Fifth, rescue of Arp1p mutants by overexpression of Arp10p is consistent with protein interactions including Jnm1p. A subset of the alleles that are suppressed by overexpression of Arp10p are also suppressed by overexpression of Jnm1p (Clark and Rose, 2005). These alleles were thought to cause a loss of dynactin function by decreasing the interaction of Jnm1p with the Arp1p filament. Rescue of this group of alleles by Arp10p overexpression is consistent with a mechanism whereby Arp10p–Jnm1p interaction counteracts the decreased interaction between Arp1p–Jnm1p.

Because Arp1p forms a two-start helical filament with rotational symmetry about the filament axis, side-binding proteins (e.g., Jnm1p and perhaps Nip100p) should bind on both faces of the filament. However, symmetrical association of dynactin subunits with the Arp1p filament is not observed (Schafer *et al.*, 1994), implying that the symmetry must be broken. Because Arp1p is itself asymmetric, the pointed and barbed ends of the filament are not equivalent, and this asymmetry must be transmitted along the filament surface. Because they are associated with the filament ends, it is reasonable to propose that Cap1p/Cap2p and Arp10p help relay the asymmetry. Nip100p requires Jnm1p for stable interaction with Arp1p (Kahana *et al.*, 1998). Therefore it is most likely that Jnm1p is the key subunit for recognition of the asymmetry. Electron microscopy data indicate that the side arm complex, including Jnm1p, projects from the filament's barbed end (Schafer *et al.*, 1994; Eckley *et al.*, 1999). Together with our evidence for interaction between Jnm1p and Arp10p, it seems likely that Jnm1p extends all along the Arp1p filament, as proposed previously (Schroer, 2004).

A Possible Pointed-End Complex in Budding Yeast

The effect of specific *arp1* mutants allows one to discriminate between associations with the barbed or the pointed end of the Arp1p filament. Only the pointed-end allele, *arp1-46*, but none of the barbed-end alleles, caused reduced Arp1p–Arp10p two-hybrid interactions. Similarly, *arp1-46* and a second pointed-end allele, *arp1-44*, caused reduced levels of Arp10p. Together with Arp10p's similarities to the established metazoan pointed-end subunit Arp11, these data argue that Arp10p associates specifically with the pointed end of the Arp1p filament.

The effect of one mutation, *arp1-35*, was surprising. The *arp1-35* Ts[−] allele exhibits wild-type Arp1p levels (Clark and Rose, 2005), and the affected residues are buried on a surface of homotypic Arp1p–Arp1p interaction. However, this mutant contained reduced levels of Arp10p, implying a reduced interaction with Arp10p. As noted above, overexpression of Arp10p also rescued this mutation. Because the residues affected by *arp1-35* are buried, and unlikely to play a direct role in the contact with Arp10p, we infer that the interaction must be sensitive to subtle changes in the Arp1p filament structure. Possibly, *arp1-35* alters homotypic Arp1p interactions, leading to “fraying” at the pointed end of the filament and reduced interactions of Arp10p with the ultimate and penultimate Arp1p subunits.

The composition of the budding yeast dynactin complex likely remains incomplete. Whereas Arp1p and Cap1/Cap2 were obvious dynactin subunits because of their sequence homology, the same was not true of Jnm1p, Nip100p, and Arp10p. Relative to metazoan and fungal dynactin complexes, budding yeast is missing pointed-end complex subunits (p62, p27, and p25) and the shoulder/sidearm subunit (p22/24). If they exist in budding yeast, they may be found by physical association with Arp10p or by genetic interactions. Budding yeast and *Neurospora crassa* dynactin have similar sedimentation coefficients (15.5S and 15S, respectively) (Kahana *et al.*, 1998; Lee *et al.*, 2001), and *N. crassa* dynactin is known to include all key subunits of dynactin, including a pointed-end complex. Thus, the smaller size of the yeast dynactin complex (relative to the 20S vertebrate complex) (Karki and Holzbaur, 1999) does not rule out the existence of a pointed-end complex.

Membrane Association of Arp1p and the Cell Wall Damage Checkpoint

The behavior of strains dependent on the activity of the hRAS–Arp1p fusion for life suggested that Arp10p inhibits the association of dynactin with the plasma membrane. The alternative hypothesis is that Arp10p interferes sterically with Ras signaling. This seems less likely because we expect that there would be multiple hRAS–Arp1p proteins and only one Arp10p in the complex. The requirements for Jnm1p and Nip100p in the Ras assay and the ability of hRAS–Arp1p to suppress *kip3Δ* synthetic lethality support the hypothesis that a hRAS–Arp1p filament is formed. Although we do not yet know the basis of the apparent change in hRAS–Arp1p plasma membrane association, it is possible that Arp10p provides a means for regulating plasma membrane association, as shown previously for the interaction of the p150^{Glued} C terminus with the Arp1p filament (Kumar *et al.*, 2001).

Metazoan dynamitin (Jnm1p) interacts with a variety of cargoes (Schroer, 2004), and the Arp1p filament and the pointed-end complex have been proposed to specify membranous cargoes (Eckley *et al.*, 1999). The interaction of the Arp1p filament with the Golgi is the best understood of the cargo associations. Arp1p interacts with a Golgi-associated spectrin isoform that in turn binds phosphatidylinositol-(4,5)-bisphosphate (PI-(4,5)-BP) via a pleckstrin homology domain (Holleran *et al.*, 1996, 2001; Muresan *et al.*, 2001). However, no spectrin homologue is evident in yeast, suggesting that if dynactin interacts with membranes, it must do so by a different mechanism. One possibility is that the large positive-charge surface of the Arp1p filament could interact directly with negative head groups of lipids (Clark and Rose, 2005). Consistent with this hypothesis, Arp10p was identified in a genome wide screen as a phosphatidylinositol-4-phosphate and PI-(4,5)-BP interacting protein (Zhu *et al.*, 2001). However, it remains to be determined whether this

interaction was direct or via an Arp10p-associated protein. It may seem counterintuitive that loss of a protein interacting with lipids would enhance membrane binding. However, the RAS–Arp1p assay is likely only monitoring association with the plasma membrane, whereas Arp10p could affect association with additional membranes.

Mutations in subunits of the *N. crassa* pointed-end complex cause enhanced dynactin membrane interaction (Lee *et al.*, 2001). In budding yeast, Arp10p may be a negative regulator of dynactin's association with the plasma membrane. Alternatively, Arp10p may positively regulate dynactin's association with some other cellular compartment (e.g., a different membrane). In that case, loss of Arp10p would reduce association with the other compartment, indirectly leading to increased levels of dynactin at the plasma membrane. In either case, our results are consistent with a protein at the pointed end playing a role in regulating dynactin's association with the membrane.

Although dynactin has been shown to be required for the cell wall checkpoint (Suzuki *et al.*, 2004), the mechanism by which cell wall damage is signaled remains to be determined. Both Jnm1p and Nip100p are required for both the cell wall checkpoint and for hRAS–Arp1p's apparent membrane association, whereas neither dynein nor its receptor Num1p is required for either function. Together, these observations make a strong case for dynactin functions independent of the well established dynein-dependent role in spindle migration. The effects of the *arp10Δ* mutation in a spindle migration assay (synthetic lethality with *kip3*) could only be detected in the context of reduced Arp1p function. In contrast, the effects of the *arp10Δ* mutation were readily detected when the assay was sensitive to the plasma membrane association of hRAS–Arp1p. Thus, it seems likely that Arp10p functions more specifically in pathways related to dynactin's membrane association and/or cell wall damage.

The reduction of hRAS–Arp1p's signaling in the presence of cell wall damage is consistent with a model in which checkpoint signaling involves either dissociation of dynactin from the membrane or sequestration in a complex where hRAS is not functional. At first blush, the observation that *arp10Δ* suppresses the effects of the *fks* mutants in the hRAS–Arp1p assay suggests that Arp10p would act downstream of the cell wall damage signal. A major shortcoming of this hypothesis is that it predicts that the *arp10Δ* mutant would be incapable of signaling cell wall damage, contrary to observation (Suzuki *et al.*, 2004).

Our observation that loss of Arp10p enhances hRAS–Arp1p signaling, in contrast to the effects of Jnm1p and Nip100, may explain why Arp10p was not identified as being required for the checkpoint (Suzuki *et al.*, 2004). If Arp10p negatively regulates Arp1p's plasma membrane association, then increased levels of membrane-associated Arp1p in the *arp10Δ* mutant might support either a normal or enhanced cell wall damage checkpoint. In fact, loss of Arp10p may cause modestly elevated levels of cell wall damage signaling (Suzuki *et al.*, 2004). In that study, 90.8% of *arp10Δ* cells arrested in the cell cycle versus 86% for wild type, although the significance of this is unclear. Although this does not necessarily imply a direct role for Arp10p in the cell wall damage checkpoint, these results suggest that Arp10p may affect the ability of Arp1p to function in the checkpoint.

CONCLUSIONS

We have shown that Arp10p is a component of budding yeast dynactin. Arp10p resides at the pointed end of the

Arp1p filament where it likely stabilizes both Arp1p–Arp1p and Arp1p–Jnm1p interactions via association with both proteins. Together, these data demonstrate that Arp10p is the functional homologue of metazoan Arp11. Moreover, dynactin associates with membranes including the plasma membrane. In vivo genetic data further suggests that dynactin's membrane association is independent of dynein but may be regulated by cell wall damage and Arp10p.

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