Dynamic phosphoregulation of the cortical actin cytoskeleton and endocytic machinery revealed by real-time chemical genetic analysis

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We used chemical genetics to control the activity of budding yeast Prk1p, which is a protein kinase that is related to mammalian GAK and AAK1, and which targets several actin regulatory proteins implicated in endocytosis. In vivo Prk1p inhibition blocked pheromone receptor endocytosis, and caused cortical actin patches to rapidly aggregate into large clumps that contained Abp1p, Sla2p, Pan1p, Sla1p, and Ent1p. Clump formation depended on Arp2p, suggesting that this phenotype might result from unregulated Arp2/3-stimulated actin assembly. Electron microscopy/immunoelectron microscopy analysis and tracking of the endocytic membrane marker FM4-64 revealed vesicles of likely endocytic origin within the actin clumps. Upon inhibitor washout, the actin clumps rapidly disassembled, and properly polarized actin patches reappeared. Our results suggest that actin clumps result from blockage at a normally transient step during which actin assembly is stimulated by endocytic proteins. Thus, we revealed tight phosphoregulation of an intrinsically dynamic, actin patch–related process, and propose that Prk1p negatively regulates the actin assembly–stimulating activity of endocytic proteins.

Introduction

Control of actin dynamics by associated proteins plays a crucial role in many biological processes including endocytosis, exocytosis, organelle inheritance, cell motility, and cell morphogenesis. F-actin in Saccharomyces cerevisiae is mainly found in three distinct structures: cables, the contractile ring, and cortical patches (Pruyne and Bretscher, 2000). Actin patches can be highly motile (0.06–1 μm/s) and undergo active turnover (for review see Engqvist-Goldstein and Drubin, 2003). They are also functionally linked to endocytosis, as many actin patch components are essential for this process, and drugs that perturb actin turnover inhibit endocytosis (for review see Engqvist-Goldstein and Drubin, 2003). Consistent with these observations, transient association between the actin cytoskeleton and endocytic sites has recently been shown to be a characteristic of caveola- and clathrin-mediated endocytosis in mammalian cells (for review see Engqvist-Goldstein and Drubin, 2003). However, the molecular mechanisms underlying actin’s involvement in endocytosis remain poorly understood.

Yeast actin-regulating kinase (Ark) 1p and Prk1p, a redundant pair of Ark family kinases, are strong candidates to directly couple the dynamic processes of actin cytoskeleton assembly and endocytosis (for review see Smythe and Ayscough, 2003). The three known in vivo targets of Prk1p, Pan1p (Eps15-related Arp2/3 activator; Zeng and Cai, 1999; Duncan et al., 2001), Ent1p (epsin-related protein; Watson et al., 2001), and Sla1p (an adaptor for Ste2p receptor endocytosis; Zeng and Cai, 1999; Howard et al., 2002), are actin patch proteins. Each of these Prk1p targets plays an important role in both endocytosis and in actin cytoskeleton regulation...
Results and discussion

Construction of prk1-analogue-sensitive (prk1-as) mutants that rapidly respond to 1NA-PP1

To determine how Prk1p functions in actin organization and endocytosis in living cells, we created analogue-sensitive mutants (Bishop et al., 2001) of Prk1p in cells that lack Ark1p. Inactivation of both kinases was necessary because either one alone is sufficient to carry out the functions related to actin organization (Cope et al., 1999) and endocytosis (unpublished data). We constructed ark1Δ prk1-as1 (with a M108G mutation) and ark1Δ prk1-as3 (with M108G and C175A mutations) strains, in which substitutions of bulky amino acids in the ATP-binding pocket of Prk1p were made to render the kinase sensitive to a PP1 analogue, 4-amino-1H-[1’-naphthyl]pyrazolo[3,4-d]pyrimidine (1NA-PP1; Bishop et al., 1998). In the absence of 1NA-PP1, both ark1Δ prk1-as1 and ark1Δ prk1-as3 mutants had growth rates and actin morphologies indistinguishable from the wild-type parent strain, and showed normal growth at 37°C.

ark1Δ prk1-as1 and ark1Δ prk1-as3 cells showed specific sensitivity to 1NA-PP1. Upon treatment of these mutants with 1NA-PP1, un polarized actin and actin clumps were observed in a dose-dependent manner (Fig. 1, A and B), indicating that inhibitor treatment mimics the phenotype seen upon loss of Ark1p and Prk1p (Cope et al., 1999). Actin cables appeared to be unaffected by inhibitor addition (Fig. 1 A; +1NA-PP1). As assessed by quantifying the percentage of cells forming actin clumps, the effect of 1NA-PP1 was saturated at 80 μM for ark1Δ prk1-as1, and at 40 μM for ark1Δ prk1-as3 (Fig. 1 B). As a further indication that ark1Δ prk1-as3 cells are more sensitive to the inhibitor, at optimal inhibitor doses, 40 μM for ark1Δ prk1-as3 and 80 μM for ark1Δ prk1-as1, ~95% of ark1Δ prk1-as3 cells formed actin clumps, whereas ~80% of ark1Δ prk1-as1 cells formed clumps (Fig. 1 B). The actin cytoskeleton of ark1Δ PRK1 cells was not affected by 40–120 μM 1NA-PP1 (unpublished data).

Next, we analyzed the in vivo phosphorylation of Ent1p, a target of Prk1p that shows a Prk1p-dependent mobility shift (Watson et al., 2001). Both ark1Δ prk1-as1 and ark1Δ prk1-as3 cells display wild-type Ent1p phosphorylation levels in the absence of 1NA-PP1 (Fig. 1 C). Addition of inhibitor for 30 min resulted in a dose-dependent inhibition of Ent1p phosphorylation in ark1Δ prk1-as1 cells (Fig. 1 D, bottom). With 80 μM inhibitor, Ent1p phosphorylation was severely inhibited by 5 min, and appeared completely inhibited by 15 min (Fig. 1 E). Ent1p phosphorylation in ark1Δ PRK1 cells was not affected by 40–120 μM 1NA-PP1 (Fig. 1 D, top). Thus, 1NA-PP1 specifically and rapidly inhibits Prk1p as kinase activity. Next, we evaluated actin organization as a function of time after Prk1p inhibition (Fig. 1 F). By 2 min, ~60% of the ark1Δ prk1-as3 cells had lost actin patch polarization and/or had actin clumps. By 20 min, the percentage of the cells with actin clumps reached saturation (~80% for ark1Δ prk1-as1, unpublished data; ~95% for ark1Δ prk1-as3, Fig. 1 F). Interestingly, at 2 min, the majority of the actin clumps in ark1Δ prk1-as1 and ark1Δ prk1-as3 cells were found in the daughter cell. At 5 min, actin clumps were mainly seen in the mother cell.

The Prk1p target Pan1p is an activator of the Arp2/3 complex. By introducing the temperature-sensitive arp2-1 mutant of an Arp2/3 complex subunit into the arch1Δ prk1-as3 background, we tested whether Arp2/3-mediated actin assembly is required for clump formation. The arp2-1 mutant shows significant endocytic defects at the permissive temperature, although it displays normal actin organization (Moreau et al., 1997). The arch1Δ prk1-as3 arp2-1 cells formed actin clumps much less efficiently (~5%, n = 100) than arch1Δ prk1-as3 cells (~95–100%, n = 100) at 25°C (Fig. 1 G). The actin clumps disappeared rapidly in response to the actin monomer sequestering drug latrunculin-A (Ayscough et al., 1997; unpublished data), suggesting that actin turnover is not severely affected by Prk1p inhibition. In total, these data support the possibility that actin clumps are the result of unregulated Arp2/3-stimulated actin assembly.

Real-time analysis of actin patch dynamics upon Prk1p inhibition

To investigate actin patch dynamics as a function of Prk1p inhibition, we performed real-time analyses of ark1Δ prk1-as1 cells expressing Abp1-GFP (Fig. 2). Similar results were obtained with ark1Δ prk1-as3 cells (unpublished data). Abp1p is a component of cortical actin patches (Drubin et al., 1988). Within 1 to 2 min of 1NA-PP1 addition to ark1Δ prk1-as1 cells, Abp1-GFP patches aggregated into clumps (Fig. 2 A and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200305077/DC1). Once formed in the daughter cell, the actin clumps invariably moved toward the bud neck, and then into the mother cell (Fig. 2 B), consistent with the observation for fixed cells (Fig. 1 F), which exhibited an increase of mother clumps and a decrease of daughter clumps during the time course. The mechanistic basis for this movement is unknown, but does not seem to involve microtubules because the process was not sensitive to the microtubule-depolymerizing drug nocodazole (unpublished data). Within 1 min of Prk1p reactivation by inhibitor washout, the clumps disassembled, and the normal polarized distribution of Abp1 patches was
Figure 1. Initial characterization of \textit{ark1Δ prk1-as} mutants. (A) To observe the actin morphology, cells were stained with rhodamine-phalloidin. The range of actin morphologies of \textit{ark1Δ prk1-as3} cells treated with 1NA-PP1 for 2 min is shown. A mock-treated cell is also shown. (B) Actin morphology of small-budded \textit{ark1Δ prk1-as1} (AS1) and \textit{ark1Δ prk1-as3} (AS3) cells was scored as a function of 1NA-PP1 concentration in a blind study \((n \geq 200\) for each sample). The cells were treated with the inhibitor for 1 h before fixation. \(\text{(C and D) } \textit{ark1Δ PRK1 (WT), ark1Δ prk1-as1, and ark1Δ prk1-as3} \) cells were cultured without 1NA-PP1 (C), or with the indicated concentration of 1NA-PP1 for 30 min (D), and then processed for anti-Ent1p Western blotting. \(\text{(E) Ent1 phosphorylation as a function of time in \textit{ark1Δ prk1-as1} cells treated with 80 \text{\mu M} 1\text{NA-PP1}. \text{(C–E) Phosphorylated (Ent1-P) and unphosphorylated (Ent1) forms of Ent1p are indicated. } \text{(F) Actin morphology of small-budded \textit{ark1Δ prk1-as3} cells in the presence of 40 \text{\mu M} 1\text{NA-PP1} at indicated time points \((n \geq 100). \text{(G) Actin morphology of \textit{ark1Δ prk1-as3} and \textit{ark1Δ prk1-as3 arp2–1} cells. The cells were mock treated or treated with 1\text{NA-PP1 at }25^\circ \text{C for 15 min before fixation. Strains: } \textit{ark1Δ PRK1, DDY2547; ark1Δ prk1-as1, DDY2595; ark1Δ prk1-as3, DDY2597; ark1Δ prk1-as3 arp2–1, DDY2610. Bars, 5 \text{ \mu m.}}\) \) \)
restored (Fig. 2 C and Video 2). Rhodamine-phalloidin staining of fixed \( \text{ark1}\Delta \text{ prk1}-as1 \) cells confirmed that F-actin undergoes reversible aggregation upon 1NA-PP1 addition (unpublished data).

The endocytic proteins Sla2p, Pan1p, Sla1p, and Ent1p, which localize at actin patches in wild-type cells, were previously shown to be present in the large actin clumps in \( \text{ark1}\Delta \text{ prk1}\Delta \) cells (Cope et al., 1999; Watson et al., 2001; Warren et al., 2002). We found that all of these proteins are also present in the inhibitor-induced clumps (Fig. 2 D for Sla2p; unpublished data for Pan1p, Sla1p, and Ent1p). The rapid and reversible actin clump formation described in Figs. 1 and 2 revealed that the wild-type actin patches are under tight regulation by protein phosphorylation. Several lines of evidence support the conclusion that actin clump formation is a direct consequence of inhibiting phosphorylation of actin patch components. First, all known in vivo targets (Pan1p, Sla1p, and Ent1p) of Prk1p are actin patch proteins. Second, Prk1p kinase is exclusively localized in cortical actin patches (Cope et al., 1999; Zeng and Cai, 1999). Third, actin clumps form rapidly upon addition of 1NA-PP1, and disappear rapidly when it is removed.

Receptor-mediated endocytic internalization is blocked upon Prk1p inhibition

Further, we tested whether Prk1p kinase activity is required for \( \alpha \)-factor internalization by its receptor, Ste2p. Because of its high sensitivity to 1NA-PP1, all subsequent analyses used the \( \text{prk1}-as3 \) allele. Without inhibitor, \( \text{ark1}\Delta \text{ prk1}-as3 \) cells show internalization kinetics indistinguishable from \( \text{ark1}\Delta \text{ PRK1} \) (Fig. 3 A). Treatment of \( \text{ark1}\Delta \text{ prk1}-as3 \) cells with 1NA-PP1 for 30 min specifically inhibited receptor internalization (Fig. 3 B). Even 120 \( \mu \)M inhibitor did not affect \( \alpha \)-factor internalization by \( \text{ark1}\Delta \text{ PRK1} \) cells (Fig. 3 B). Additionally, a kinase-dead mutant (\( \text{ark1}\Delta \text{ prk1}^{D159A} \)) also showed a severe block of receptor internalization (Fig. 3 C). Thus, the inhibition of Prk1p kinase activity profoundly blocks the internalization step of receptor-mediated endocytosis.

Association of endocytic membranes with actin upon Prk1p inhibition

Next, we investigated the fate of the endocytic membrane marker FM4-64 (Vida and Emr, 1995) in inhibitor-treated \( \text{ark1}\Delta \text{ prk1}-as3 \) cells (Fig. 4). FM4-64 was first incorporated
into the plasma membrane (Fig. 4 A, 0 min). In mock-treated \textit{ark1Δ prk1Δ-as3} or wild-type cells, this dye is later found in endosomes (Fig. 4 A, top, 5 min) and finally accumulates in vacuoles (Vida and Emr, 1995; unpublished data). In mock-treated cells, the endosomes seen at 5 min do not show significant colocalization with Abp1p (Fig. 4 A, bottom). Next, we treated \textit{ark1Δ prk1Δ-as3} cells simultaneously with FM4-64 and 1NA-PP1. At 5 min, we found that FM4-64 staining colocalized with actin clumps (Fig. 4 A, bottom). The FM4-64 dye in actin clumps was later transported to vacuoles (after 15–20 min), although the kinetics were delayed compared with the wild type (10 min; unpublished data). These observations suggest that the block of Prk1p activity leads to accumulation of an endocytic compartment that associates with actin.

Next, we examined \textit{ark1Δ prk1Δ-as3} (Fig. 4, C–H and K–N) and inhibitor-treated \textit{ark1Δ prk1Δ-as3} cells (Fig. 4 I and J for cells treated for 10 min; also see Fig. S1) by conventional EM. To better visualize actin filaments, we first observed cells post-treated with tannic acid (Fig. 4, B–J). Compared with wild-type cells that showed no remarkable morphology (Fig. 4 B), both \textit{ark1Δ prk1Δ} and inhibitor-treated \textit{ark1Δ prk1-as3} cells showed clustered vesicles of \textasciitilde{}100 nm (arrows) in the area where microfilaments (arrowheads) are observed (Fig. 4, C–J, dashed area; also see Fig. S1). Next, we observed \textit{ark1Δ prk1Δ} cells processed by high pressure freezing followed by freeze substitution, and also detected accumulation of \textasciitilde{}100-nm vesicles (Fig. 4, K–N, arrows). With this procedure, the vesicles are seen within areas that exclude ribosomes (Fig. 4 K, dashed area), and they sometimes appear to have electron-dense coats (Fig. 4 M, left arrow; Fig. 4 N, arrow). The colocalization of vesicles with microfilaments and ribosome exclusion areas suggested that the vesicles might reside within actin clumps. Therefore, we used immuno-EM to determine if this was indeed the case (Fig. 4, O–R). In indirect immunolabeling for actin on ultrathin cryosections of fixed \textit{ark1Δ prk1Δ} cells, 10-nm gold particles were seen to localize to slightly electron-dense areas (Fig. 4 O, dashed area). These immunogold-labeled actin clumps were seen to contain \textasciitilde{}100-nm vesicles similar to those seen using conventional EM (Fig. 4, O–Q, dashed boxes). Thus, we conclude that the block of the Ark kinases leads to the accumulation of actin and actin-associated vesicles. Additionally, in double-immunogold labeling for Slal-GFP (5-nm gold) and actin (10-nm gold), Slal-containing vesicles were occasionally detected within the actin clumps (Fig. 4 R).

In this work, we examined the rapid and acute effects of Prk1p kinase inhibition and reactivation by applying a chemical genetics approach. We showed that abnormal actin clumps formed and disappeared within 1 to 2 min of Prk1p inhibition and reactivation, respectively. Further, we showed that the actin clumps contain endocytic proteins and \textasciitilde{}100-nm vesicles. We propose that Prk1p directly regulates the coupling between actin assembly and endocytosis by promoting disassembly and/or inactivation of an early endocytic complex that stimulates actin assembly (Fig. 5 A). When Prk1p is inhibited, this complex is stabilized, and actin assembly continues to be stimulated by endocytic proteins such as the Prk1p target Pan1p and the associated Arp2/3 complex (Zeng and Cai, 1999; Duncan et al., 2001), and/or other targets including Slalp and Ent1p (Watson et al., 2001; Zeng et al., 2001; Fig. 5 B). In mammalian cells, the \textmu{}2 subunit of AP2 is phosphorylated by AAK1 at Thr-156 (ITSQVT156G) (Ricotta et al., 2002). However, the budding yeast AP2 homologue, Apm4p (Huang et al., 1999), is not important for receptor internalization and does not contain potential Prk1p-phosphorylation motifs similar to (L/ixxQxTG). Rather, our genetic experiment showing that Arp2p is required for clump formation supports the idea that Arp2/3-mediated actin assembly is negatively regulated by Prk1p, potentially via phosphorylation of the Arp2/3 ac-
Figure 4. Actin-associated membrane accumulation upon inhibition of Prk1p activity. (A) FM4-64 labeling of ark1Δ prk1-as3 cells expressing Abp1-GFP. Cells were treated with media containing FM4-64 and 1NA-PP1 in a flow chamber. FM4-64 (red) and Abp1-GFP (green) were visualized at 0 and 5 min. Mock-treated cells are also shown. (B) A wild-type cell shows normal, unremarkable morphology. Bars: (A) 5 μm; (B) 1 μm; (C) 0.2 μm; (D) 0.5 μm; (E–I) 0.1 μm; (K) 0.5 μm; (L–R) 0.1 μm.

Materials and methods

Media, growth conditions, and materials

Yeast strains were grown in standard rich media (YPD) or synthetic media (SD) supplemented with the appropriate amino acids. 1NA-PP1 was synthesized and handled as described previously (Bishop et al., 1998). ark1Δ prk1-as1 cells were treated with 80 μM inhibitor and ark1Δ prk1-as3 cells with 40 μM inhibitor in SD-based media unless indicated otherwise. All the yeast strains were cultured at 25°C, except for DDDY904 and ark1Δ prk1Δ, which were cultured at 30°C.

Strains and plasmids

The yeast strains are listed in Table I. prk1Δ::PRK1::URA3 and prk1Δ::prk1::URA3 integration plasmids were created as follows: First, a NotI site was introduced 249 bp upstream of the PRK1 ORF. The mutated KpnI/SacI PRK1 fragment was cloned into pBlueScript® II SK, and the PRK1 fragment was marked with URA3 at NotI to create a plasmid pDD877. prk1-as1 (M108G), prk1-as3 (M108G, C175A), and prk1D159A mutations were created in pDD877. The URA3-marked, PRK1-containing fragments were excised from the integration plasmids, and replaced the prk1Δ::LEU2 locus. These strains were each crossed with an ark1Δ strain, and the diploids were sporulated to obtain ark1Δ prk1 mutants. Analogue-sensitive Prk1 mutant proteins were expressed at normal levels, but Prk1D159A was expressed at only 20–30% of normal levels at 25°C. The gene deletions were created as described previously (Cope et al., 1999). Functional GFP and CFP tags were integrated at the COOH terminus of Abp1p and Sla1p as described previously (Warren et al., 2002). To create Sla2-YFP, five alanines were introduced at the junction between the SLA2 ORF and YFP using pDH5 (from the Yeast Resource Center, Seattle, WA). The Sla2-YFP strain has growth properties that are indistinguishable from the wild type. pDD890 expresses Abp1-GFP from pRB2139 (Doyle and Botstein, 1996) on pRS317.
Figure 5. Model for Ark-kinase function in endocytosis. (A) In wild-type cells, Prk1p promotes proper disassembly of the actin-associated endocytic complex. Phosphatase(s) might be responsible for reformation and/or activation of the endocytic complex. Ark1p, a closely related homologue of Prk1p, is likely to perform an overlapping function. (B) When Ark1p and Prk1p are inhibited, vesicles associated with actin filaments and endocytic proteins accumulate. See text for further discussion.

### Fluorescent microscopy

F-actin was stained as described previously (Cope et al., 1999). FM4-64 was used at a concentration of 8 μM in SD-based media. For live imaging, cells were attached to 50 μg/ml concanavalin A-coated long coverslips (24 x 50 mm). A flow chamber was made by mounting a small coverslip (18 x 18 mm) on top of the long coverslip with grease and small pieces of coverslips as spacers. Two sides were left unsealed so that media could be exchanged. Cells were viewed using a microscope (model TE300, Nikon) equipped with a 100× objective, NA 1.4, and a cooled CCD camera (Hamamatsu). Phase-3 software (Phase-3 Imaging Systems) was used for visualization of rhodamine, GFP, and FM4-64. In a CFP–YFP colocalization study, cells were photographed by rapidly alternating excitation/emission filters (IP4 filter sets; Chroma Technology Corp.). Filter wheels (Lambda 10–2; Sutter Instrument Co.) were mounted on the excitation and emission ports of the microscope and were controlled with MetaMorph® software (Universal Imaging Corp.).

### Table 1. Yeast strains

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*a*Derived from DDY426.  
*b*Derived from DDY1102.  
*c*cgHSF and cgLEU2 indicate Candida glabrata HIS3 and LEU2 genes, respectively.  
*d*arp2–1 allele (Moreau et al., 1997) was crossed into our strain background.

### Protein and immunological techniques

To obtain yeast whole-cell lysates, the cells were lysed with glass beads in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, protease inhibitor cocktail, 50 mM NaN3, 0.2 mM sodium orthovanadate, 25 mM β-glycerophosphate, 1 μM cyscoporin A, 4% (w/v) glutaraldehyde, 50 μg of the lysate was loaded per lane. For the time-course experiment, 2-OD cells were harvested and resuspended in 50 μl lysis buffer (50 mM Tris, pH 6.8, 2% SDS, 100 mM DTT, 8% glycerol, 0.02% BPB, protease inhibitor cocktail, 50 mM NaF, 0.2 mM sodium orthovanadate, 25 mM β-glycerophosphate, and 1 μM cyscoporin A). The suspension was boiled for 3 min, and then lysed with glass beads for 2 min, followed by 1 min of boiling. 100 μl of the SDS-PAGE buffer was finally added to the lysate. 10
μl of the final supernatant was loaded per lane. Anti-Ent1p antibody (Watson et al., 2001) was used at 1:10,000 dilution for Western blotting.

α-factor uptake assay

35S-labeled α-factor was prepared as described in Howard et al. (2002). The α-factor uptake assay was performed at 25°C based on a continuous incubation protocol (Dulic et al., 1991) with modifications as follows: cells were grown in SD, harvested by centrifugation, and resuspended in internalization media (SD media with 0.5% casamino acid and 1% BSA). Then the cells were mixed with an equal volume of SD media containing 2× concentration of 1NA-PP1. After incubation with 1NA-PP1 for 30 min, 30,000 cpm/100 μl 35S-labeled α-factor was added at time zero. At the indicated time points, aliquots were withdrawn and diluted in ice-cold buffer at pH 6.0 (total α-factor) or pH 1.1 (internalized α-factor). The samples were then filtered, and radioactivity was measured in a scintillation counter. The results were expressed as the ratio of pH 1.1 cpm/pH 6.0 cpm for each time point to represent the percentage of internalization.

Online supplemental material

Video 1 and Video 2 show ark1Δ prk1-as1 cells expressing Abp1-GFP. The representative frames of the movies are shown in Fig. 2 A and Fig. 2 C. Fig. S1 shows EM of ark1Δ prk1-as3 cells post-treated with tannic acid. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200305077/DC1.

We thank James Howard and Gregory Payne for technical assistance and helpful discussion regarding the α-factor uptake assay; Barbara Winsor (Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France) for providing the arp2-1 strain and Anthony Bretscher for providing the anti-actin antibody. We also thank the members in Drubin lab for helpful discussions, and Daria Siekhaus and Adam Martin for critical reading of the manuscript.

This work was supported by National Institutes of Health grants to D.G. Drubin (GM42759 and GM50399) and to K.M. Shokat (AI44009); an NIH grant (GM60979) and a Burroughs Wellcome Fund New Investigator Award to B. Wendland; and a National Science Foundation grant (DBI 0099705) to B. Wendland and J.M. McCaffery.

Submitted: 15 May 2003
Accepted: 21 July 2003

References


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