Phosphorylation-dependent inhibition of Cdc42 GEF Gef1 by 14-3-3 protein Rad24 spatially regulates Cdc42 GTPase activity and oscillatory dynamics during cell morphogenesis

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ABSTRACT Active Cdc42 GTPase, a key regulator of cell polarity, displays oscillatory dynamics that are anticorrelated at the two cell tips in fission yeast. Anticorrelation suggests competition for active Cdc42 or for its effectors. Here we show how 14-3-3 protein Rad24 associates with Cdc42 guanine exchange factor (GEF) Gef1, limiting Gef1 availability to promote Cdc42 activation. Phosphorylation of Gef1 by conserved NDR kinase Orb6 promotes Gef1 binding to Rad24. Loss of Rad24–Gef1 interaction increases Gef1 protein localization and Cdc42 activation at the cell tips and reduces the anticorrelation of active Cdc42 oscillations. Increased Cdc42 activation promotes precocious bipolar growth activation, bypassing the normal requirement for an intact microtubule cytoskeleton and for microtubule-dependent polarity landmark Tea4-PP1. Further, increased Cdc42 activation by Gef1 widens cell diameter and alters tip curvature, countering the effects of Cdc42 GTPase-activating protein Rga4. The respective levels of Gef1 and Rga4 proteins at the membrane define dynamically the growing area at each cell tip. Our findings show how the 14-3-3 protein Rad24 modulates the availability of Cdc42 GEF Gef1, a homologue of mammalian Cdc42 GEF DNMBP/TUBA, to spatially control Cdc42 GTPase activity and promote cell polarization and cell shape emergence.

INTRODUCTION Establishment of cell polarity and maintenance of cell shape are fundamental cellular processes in development and cell differentiation. Defects in cell morphogenesis are linked to diseases such as cancer, developmental defects, and neuronal disorders (Yoshimura et al., 2006; Tanos and Rodriguez-Boulan, 2008; Tahirovic and Bradke, 2009; Carroll and Das, 2011; Yates and Dean, 2011). The conserved GTPase Cdc42 is a key regulator of polarized cell growth (Johnson, 1999; Etienne-Manneville, 2004). Active, GTP-bound Cdc42 promotes asymmetric actin cytoskeleton organization, membrane recycling, and vesicle secretion at the site of polarized cell growth (Pruyne and Bretscher, 2000; Heasman and Ridley, 2008; Harris and Tepass, 2010; Perez and Rincon, 2010; Perez and Rincon, 2010; Martin and Arkowitz, 2014). Although substantial progress has been made in our understanding of the cellular processes underpinning cell polarization (Suzuki and Ohno, 2006; Slaughter et al., 2009; Das and Verde, 2013; Wu and Lew, 2013; Halloupi and McCaffrey, 2015), the molecular mechanisms that spatially regulate Cdc42 activity during cell morphogenesis are still poorly understood.

Fission yeast is a particularly amenable model system for studies of cell morphogenesis because it grows in a polarized manner from two cell tips, or cell ends, and displays a well-defined rod shape. Cellular dimensions are uniform and vary little from cell to cell. Cell diameter is maintained at a constant size during cell growth and...
division (Streiblova and Wolf, 1972), and during the cell cycle, new daughter cells start growth in a monopolar manner from the old end that existed in the previous cell cycle (old-end take off [OETO]; Streiblova and Wolf, 1972; Mitchison and Nurse, 1985). On exit from G1, cells activate bipolar growth from the new end (new-end take off [NETO]) once a critical cell size is reached (Mitchison and Nurse, 1985). Microtubules also play an important role in the activation of bipolar growth by delivering the Tea1–Tea4 scaffold complex, which functions as a polarity landmark, recruiting type 1 phosphatase (PP1) to the cell tips (Martin and Arkowitz, 2014). It is poorly understood how bipolar growth activation is coordinated with cell size and modulated by microtubules and what mechanisms regulate cellular dimensions.

Active Cdc42 displays oscillatory dynamics at the cell tips in fission yeast, revealing the presence of positive and negative feedback controls (Das et al., 2012). Cdc42 oscillatory dynamics has also been observed in other systems (Carlin et al., 2011; Howell et al., 2012; Kuo et al., 2014). In fission yeast cells, oscillations are anticorrelated at the cell tips; when Cdc42 activity is high at one tip, it is lower at the other, indicating competition for active Cdc42 or its regulators (Das et al., 2012). Our findings indicate that Cdc42 dynamics and distribution are governed by a cell-scale system: varying the levels of Cdc42 regulatory factors changes the behavior of the system, altering the symmetry of Cdc42 distribution at the two cell tips. Because Cdc42 is a key determinant of cell growth, the pattern of bipolar growth activation also changes (Das et al., 2012). In particular, Cdc42 dynamics and distribution at the two cell tips are sensitive to the protein level of the Cdc42 guanine nucleotide exchange factor (GEF) Gef1, suggesting that Gef1 availability is limiting under normal conditions.

We previously reported that the conserved nuclear Dbf2-related (NDR) kinase Orb6 has a function in the spatial distribution of active Cdc42 at the cell membrane of fission yeast (Das et al., 2009). Orb6 is part of the NDR/large tumor suppressor (LATS) family of kinases, with conserved functions in the control of morphogenesis and cell proliferation in eukaryotes (Hergovich et al., 2006). NDR kinases have a role in the control of neuronal differentiation (Zallen et al., 2000; Emoto et al., 2004, 2006; Gallegos and Bargmann, 2004; Zinn, 2004) and, in humans, can function as tumor suppressors (Hergovich et al., 2008; Comnís et al., 2011). In fission yeast, Orb6 kinase is required for maintenance of cell polarity (Verde et al., 1995, 1998) and prevention of ectopic activation of Cdc42 GTPase (Das et al., 2009).

Here we show that the NDR kinase Orb6 governs Cdc42 dynamics and distribution by negatively regulating the Cdc42 GEF Gef1. We show that Gef1 is a Bin-amphiphysin-Rvs (BAR) domain–containing GEF that is related to mammalian TUBA/DNMBP, a Cdc42 GEF with a role in kidney cell polarization (Bryant et al., 2010; Zuo et al., 2011). Variations in the TUBA/dynamin-binding protein (DNMBP) gene sequence have been linked to Alzheimer’s disease in certain ethnic groups (Kuwano et al., 2006; Bettens et al., 2009). Orb6 kinase phosphorylates Gef1 to generate a 14-3-3–binding site, leading to Gef1 sequestration in the cytoplasm. Consistent with a role for Orb6 kinase in limiting Gef1 availability, loss of Orb6-dependent phosphorylation of Gef1 leads to increasingly symmetrical GTP-Cdc42 distribution at the cell tips and precocious bipolar growth activation, which bypasses the requirement for the microtubule cytoskeleton and the microtubule-dependent landmark Tea4-PP1 phosphatase. Further, loss of Gef1 phosphorylation increases cell width. We find that increasing levels of Cdc42 GTPase-activating protein (GAP) Rga4 can suppress these effects and show that Gef1 and Rga4 cooperate in modulating GTP–Cdc42 distribution.

Our findings describe a system that dynamically regulates the shape of Cdc42 distribution at each cell tip, finely scaling up and down the area of cell growth, modulating the extent of cell polarization, and controlling the pattern of cell growth.

**RESULTS**

**Loss of Orb6 kinase activity leads to increased cortical levels of the Cdc42 GEF Gef1 protein**

We previously reported that Orb6 kinase spatially regulates Cdc42 GTPase distribution, restricting Cdc42 activation to the cell tips (Das et al., 2009). Decreasing Orb6 kinase activity using an inhibitor-sensitive allele of orb6, orb6-as2, leads to loss of cell polarity and ectopic activation of Cdc42 at the cell sides that is dependent on the presence of Gef1 (Das et al., 2009). Here we report that loss of Orb6 kinase, in addition to inducing ectopic localization of Gef1 to the cell sides (Figure 1Aa; Das et al., 2009), also strongly increases the cortical levels of Gef1 (Figure 1, Ad and B) compared with controls (Figure 1, A, a–c, and B) within 15 min after addition of the inhibitor 1-Phosphatase (PH) domain precedes the Dbl homology (DH) domain (Rossman et al., 2003), Gef1 contains a C-terminal BAR domain (Figure 1D) as determined by protein fold recognition software (PHYRE2; Supplemental Figure S1A). Of interest, another Schizosaccharomyces pombe Rho GEF, Gef3, has also been found to contain a BAR domain before the DH domain. Gef3 interacts with Rho3 and the septin complex and plays a role in cytokinesis (Muñoz et al., 2014; Wang et al., 2015). Sequence comparison of Gef1 with different species using Ensembl/Fungi indicates that Gef1 is an orthologue of the Cdc42 GEF TUBA/DNMBP found in mammals and nematodes (Salazar et al., 2003; Figure 1D and Supplemental Figure S1B). Similar to Gef1, in the TUBA protein, the DH domain is not preceded by a PH domain but instead is followed by a BAR domain (Figure 1D; Salazar et al., 2003). In mammalian cells, TUBA is believed to perform a specialized role in promoting Cdc42-dependent exocytosis (Bryant et al., 2010; Zuo et al., 2011).

**Cortical localization of Gef1 is negatively regulated by association with the 14-3-3 protein Rad24**

The different domain structure of Gef1 required structure–function analysis. To analyze the function of the different domains of Gef1, we generated nested deletion mutations in the gef1 gene (Figure 1D) and analyzed their effect on the localization of the corresponding Gef1-3YFP mutant proteins. Under normal conditions, the full-length Gef1-3YFP displays a discernible but faint localization to the cell tips and cell septum (Figure 1Ea). Deletion of the N-terminal domain (amino acids [aa] 1–314) of the Gef1 protein (gef1ΔN-term-3YFP [aa315–753]; Figure 1D) leads to complete loss of Gef1 localization from the cell cortex (Figure 1Eb). Consistent with loss of Gef1 function in the control of polarization, deletion of the N-terminal domain of the Gef1 protein leads to increased asymmetry of growth.
FIGURE 1: Phosphorylation of Gef1 N-terminus by Orb6 kinase. (A) Gef1-3YFP localization in orb6+ (a, b) and analogue-sensitive orb6-as2 mutants (c, d) treated with DMSO (a, c) or 50 μM 1-Na-PP1 inhibitor (b, d) for 15 min. Bar, 5 μm. (B) Quantification of the fraction of cells with ectopic Gef1-3YFP localization in control and orb6-as2 cells in the presence and absence of 50 μM 1-Na-PP1. (C) In vitro phosphorylation of bacterially expressed N-terminal Gef1 (wild type and S112A mutant) by Mob2-bound Orb6 kinase, immunoprecipitated from cell extracts of wild-type and orb6 mutants (as in Das et al., 2007). (D) Sequence alignment of S. pombe Gef1 with human TUBA/DNMBP. Gef1 protein contains a proline-rich Src homology 3 domain (SH3)-binding site at the N-terminus, a GEF domain, and a C-terminus BAR domain. Also shown is a schematic of the deletion mutants of Gef1 analyzed in E. (E) Cortical localization of Gef1 protein (arrows); (a) control Gef1-3YFP, (b) Gef1-ΔNterm-3YFP, and Gef1-ΔBAR-3YFP (c). Bar, 5 μm.
(Supplemental Figure S1C) in which 75% of gef1-ΔN-term-3YFP cells are monopolar, similar to gef1Δ mutants (71%; see gef1-3YFP control cells, 36%). Conversely, deletion of the BAR domain (aa 507–753; gef1-ΔBAR-3YFP; Figure 1D) does not affect the localization of Gef1-ΔBAR-3YFP (Figure 1E). However, even though the Gef1-BAR-3YFP mutant protein is correctly localized to the cell tips and site of cell division, the gef1-BAR-Δ3YFP mutant cells still display polarization defects (65% monopolar), indicating that the BAR domain is essential for Gef1 function (Supplemental Figure S1C). Protein levels of Gef1-BAR-3YFP and Gef1-ΔN-term-3YFP were comparable to full-length Gef1-3YFP in these experimental conditions (Supplemental Figure S1, D and E). Thus our observations indicate that the N-terminal domain (aa 1–314) of Gef1 is essential for its localization to the cell cortex, and the BAR domain is essential for Gef1 function but not localization.

Previous studies with the Saccharomyces cerevisiae NDR/Orb6 orthologue, CBK1 kinase, and with mammalian NDR/LATS family kinases identified phosphorylation consensus sequences that are consistent with the pattern Hx[RKH][xST] (Hao et al., 2008; Mazanka et al., 2008). The Gef1 protein has a similar site on its N-terminal region, 107–112 (HRRFFS; Figure 1B, red arrow). Thus we bacterially expressed the N-terminal region of Gef1 (ΔCterm: aa 1–314; Figure 1C) and used this fragment as in vitro substrate for Orb6 kinase immunoprecipitated from yeast cells (Wiley et al., 2003). By mass spectrometry analysis, we determined that a serine residue at position 112 in the N-terminus is directly phosphorylated by Orb6. Consistent with this finding, mutation of the Gef1-S112 to alanine prevents phosphorylation (Figure 1C). We then observed that phosphorylation of serine 112 generates a putative binding site for 14-3-3 proteins. These proteins are highly conserved regulatory proteins that dimerize to bind to a wide range of signaling molecules (Hermeking and Benzing, 2006). They typically interact with ligands with the recognition motif RxS, where serine is phosphorylated (Yaffe et al., 1997). In S. pombe, there are two 14-3-3 proteins, Rad24 and Rad25, which are involved in the DNA damage checkpoint, regulation of transcription, meiosis, and cytokinesis (Kitamura et al., 2001; Ozoe et al., 2002; Mishra et al., 2005; Chen et al., 2008).

A common role of 14-3-3 proteins is sequestration of their targets from the normal site of function (Hermeking and Benzing, 2006). We hypothesized that Orb6 phosphorylates Gef1 to promote 14-3-3 binding and sequestration of Gef1 in the cytoplasm. We further predicted that loss of 14-3-3 proteins would lead to increased cortical localization of Gef1 and morphological changes. Consistently, we found that with deletion of the 14-3-3 protein, rad24Δ mutant cells are wider and rounder (Figure 2Ad) than wild-type cells (Figure 2Aa), whereas cells carrying the deletion of rad25, encoding the second 14-3-3 fission yeast protein, do not display a significant change in morphology (Hayles et al., 2013). In rad24Δ cells, Gef1-3YFP localization is increased at the cell tips and is often ectopically localized to the cell sides compared with rad24- control cells (Figure 2, A, b and e, and B). Conversely, the other Cdc42 GEF, Scd1, is normally localized to the cell tips in both rad24Δ and rad24Δ cells (Figure 2A, c and f). Consistent with a direct physical interaction between Gef1 and Rad24, both Gef1-3YFP protein (Figure 2C) and Gef1-HA protein (Supplemental Figure S2A) from S. pombe cell extract expresses Orb6 kinase copurify with bacterially expressed glutathione S-transferase (GST)–Rad24. Further, we found that the interaction of Gef1-3YFP with GST-Rad24 is reduced by inhibition of Orb6-as2 kinase with 1-Na-PP1 (Figure 2D).

Finally, supporting the idea that the morphology defect of rad24Δ mutant is mediated by increased Gef1 cortical localization, we found that deletion of the gef1 gene rescues the altered cell shape of rad24Δ cells (Figure 2Ed). The cell diameter of the gef1Δrad24Δ double mutant is restored more closely to wild-type cell dimensions than is that of rad24Δ cells (Figure 2E and Supplemental Figure S2B). Note that since the gef1Δrad24Δ double mutant is not completely restored to wild-type dimensions (Supplemental Figure S2B), it is likely that Rad24 has additional functions in the control of morphogenesis that are not dependent on Gef1.

Overall these findings indicate that Rad24 physically interacts with Gef1 and disruption of the Gef1–Rad24 interaction results in increased cortical localization of Gef1 and ectopic Cdc42 activation.

**Loss of Gef1 phosphorylation alters Cdc42 dynamics, leading to increased GTP-Cdc42 symmetry and precarious activation of bipolar growth**

Our results suggest that phosphorylation of Gef1S112 mediates Gef1 sequestration to the cytoplasm via an interaction with Rad24. Thus we predicted that loss of phosphorylation at S112 should inhibit the Gef1–Rad24 interaction, resulting in increased cortical localization of Gef1. To test this hypothesis, we substituted serine with alanine at position 112 by site-directed mutagenesis to produce a nonphosphorylatable hemagglutinin (HA)-tagged and 3YFP-tagged form of Gef1 expressed at endogenous levels in a gef1Δ mutant. GST-Rad24 pull-down assays indicate that HA-tagged Gef1S112A fails to bind Rad24 protein (Figure 3C). Further, we observed increased cortical localization of Gef1S112A-3YFP compared with wild-type Gef1-3YFP (Figure 3A, a and b). Quantification of Gef1 cortical levels indicated a 10-fold increase of cortical Gef1 in gef1S112A (Figure 3B). We did not observe localization of Gef1S112A-3YFP to the cell sides (Figure 3A), suggesting that Rad24 and Orb6 kinase have additional targets that restrict Gef1 localization to the cell tips. Consistent with this idea, Gef1S112A-3YFP localized to the cell sides upon Orb6 kinase inhibition, similar to Gef1-3YFP (Figure 3Dd). On average, the percentage of cells with ectopic localization of Gef1 was similar in gef1S112A mutants and gef1Δ cells upon Orb6 kinase inhibition (Figure 3E).

We found that the S112A substitution did not change overall protein levels of either Gef1S112A-HA or Gef1-S112A-3YFP compared with Gef1-HA or Gef1-3YFP proteins (Supplemental Figure S3, A and B). To test whether the increased levels of cortical Gef1S112A corresponded to increased activation of Cdc42, we performed a GST–Cdc42/Rac interactive binding (CRIB) pull-down assay (Coll et al., 2003) in gef1Δ and gef1S112A strains. We found a twofold increase in Cdc42-GTP levels in the gef1S112A mutant compared with gef1Δ cells (Supplemental Figure S3C).

We then tested the effects of the gef1S112A mutation on the distribution of active Cdc42 GTPase, using CRIB–green fluorescent protein (GFP), a fluorescent reporter that binds to GTP-Cdc42 (Tatebe et al., 2008). We previously showed that in small wild-type monopolar cells, the majority of CRIB-GFP is localized at one end, as shown by a heat map of CRIB-GFP tip fractions in cells of various cell sizes (Das et al., 2012; Figure 4B). As the cell grows, CRIB-GFP distribution at the two tips becomes more symmetrical (Das et al., 2012; Figure 4, A and B). Gef1 overexpression leads to increased CRIB-GFP symmetry (Das et al., 2012). Thus we tested whether this effect would also be observable when cortical levels of Gef1 were increased by the gef1S112A mutation. Indeed, we found that in gef1S112A mutants, even small cells show increased CRIB-GFP symmetry (Figure 4B).

Next we studied GTP-Cdc42 dynamics in control and gef1S112A mutants by following CRIB-GFP localization over time. In wild-type cells, active Cdc42 displays oscillatory dynamics, which is anticorrelated at the two cell tips (Das et al., 2012). Anticorrelation of Cdc42...
FIGURE 2: The 14-3-3 protein Rad24 physically and functionally interacts with Gef1. (A) rad24Δ mutant cells are spherical and show ectopic localization of Gef1-3YFP. Calcofluor staining (a, d), Gef1-3YFP (b, e), and Scd1-GFP (c, f) in control cells (a–c), or rad24Δ cells (d–f). Bar, 5 μm. (B) Quantification of the percentage of cells with ectopic Gef1-3YFP localization in wild-type vs. rad24Δ cells from the experiment shown in A. (C) Endogenously tagged Gef1-3YFP copurifies with bacterially expressed GST-Rad24 but not with GST alone. (D) Physical interaction between endogenously expressed Gef1-3YFP and bacterially expressed GST-Rad24 is reduced upon inhibition of Orb6-as2 with 50 μM 1-Na-PP1 compared with DMSO treatment (lanes 7 and 8) but remains unchanged in wild-type cells in the presence or absence of the inhibitor (lanes 5 and 6). GST-only control is shown in lanes 1–4. (E) Spherical shape phenotype of rad24Δ (c) mutants is rescued in gef1Δ rad24Δ (d) double-deletion mutants. (a) Control cells; (b) gef1Δ cells. Cells were stained with Calcofluor. Bar, 5 μm.
FIGURE 3: Phosphorylation at position S112 in Gef1 regulates Gef1 localization. (A) Cortical localization of Gef1-3YFP increases in gef1S112A mutants (a) compared with control gef1+ cells (b). Calcofluor-stained gef1S112A mutant cells (d) show increased cell width compared with control cells (c). Bar, 5 μm. (B) Quantification of Gef1 cortical localization in A. (C) Endogenously tagged Gef1-HA copurifies with bacterially expressed GST-Rad24, whereas Gef1S112A-HA does not. (D) Inhibition of Orb6-as2 kinase with 50 μM 1-Na-PP1 increases cortical localization of both Gef1-3YFP and Gef1S112A-3YFP compared with DMSO treatment. Bar, 10 μm. (E) Quantification of the percentage of cells with ectopic Gef1-3YFP or Gef1S112A-3YFP upon treatment with DMSO (time, 0 min) or 1-Na-PP1 (time, 5, 10, 15, and 60 min).
oscillations is sensitive to the protein level of Gef1 (Das et al., 2012). Thus we quantified the cross-correlation coefficient of CRIB-GFP oscillations (Figure 4E) in cells expressing Gef1-HA (Figure 4C) and Gef1S112A-HA (Figure 4D) from the endogenous gef1 promoter. A cross-correlation coefficient of −1 indicates absolute anticorrelation, whereas a cross-correlation coefficient of +1 indicates absolute oscillations is sensitive to the protein level of Gef1 (Das et al., 2012). Thus we quantified the cross-correlation coefficient of CRIB-GFP oscillations (Figure 4E) in cells expressing Gef1-HA (Figure 4C) and Gef1S112A-HA (Figure 4D) from the endogenous gef1 promoter. A cross-correlation coefficient of −1 indicates absolute anticorrelation, whereas a cross-correlation coefficient of +1 indicates absolute
correlation. A coefficient value of 0 indicates lack of correlation. As
expected, control Gef1-HA cells showed anticorrelation of CRIB-GFP oscillatory dynamics between the two ends (as indicated by a negative cross-correlation coefficient of $-0.3 \pm 0.13$; Figure 4, C and E). However, CRIB-GFP anticorrelation in gef1S112A-∆A mutants was reduced, approaching the value of 0 (cross-correlation coefficient of $-0.08 \pm 0.11$; Figure 4, D and E). Therefore our results indicate that loss of Gef1 interaction with Rad24 leads to an alteration of Cdc42 dynamics in the cell.

In fission yeast cells, growth occurs from the old end immediately after cell division by a process called OETO (Mitchison and Nurse, 1985). The new end generated as a result of cell division starts growth only when the cell attains a size of at least 9 μm in length by a process called NETO (Mitchison and Nurse, 1985). In gef1Δ mutants, NETO is delayed, resulting in an increased percentage of monopolar cells (Coll et al., 2003; Das et al., 2012). We previously proposed that NETO is linked to a progressive alleviation of competition for active Cdc42 (Das et al., 2012). Thus we tested whether increased symmetry of GTP-Cdc42 at the cell tips promotes bipolar growth in gef1S112A mutant cells. Indeed, we found that gef1S112A mutants display an increased percentage of bipolar cells compared with wild-type cells (Figure 4F). Furthermore, in gef1S112A mutants, NETO occurs soon after cell separation, roughly at the same time as OETO (Figure 4, H and J), whereas NETO is delayed in wild-type cells (Figure 4, G and I; Mitchison and Nurse, 1985). On average, in gef1S112A mutants, NETO occurs 7 ± 11.7 min after cell separation, whereas in wild-type cells, NETO occurs 41.5 ± 17.7 min after cell separation ($n = 20$, $p < 0.0001$). Consistent with the idea that NETO occurs precociously and is not due to an alteration of overall cell size, the calculated average cell volume of gef1S112A cells undergoing NETO is smaller (76 ± 18.7 μm$^3$) than that of control gef1+ cells undergoing NETO (96 ± 8.4 μm$^3$; $p < 0.005$). Thus these results indicate that limiting Gef1 localization to the cell tips through a 14-3-3 interactionpromotes competition for active Cdc42 and delays the timing of NETO.

**Loss of Gef1 phosphorylation suppresses morphological defects associated with microtubule disruption or loss of Tea4-dependent PP1**

The microtubule cytoskeleton has an important role in fission yeast cell polarity and the activation of bipolar cell growth (NETO) by delivering the Tea1–Tea4 complex protein, which localizes to the microtubule plus ends and marks the cell tips (Martin and Arkowitz, 2014). The Tea1–Tea4 complex promotes microtubule-induced polarized cell growth in fission yeast by recruiting the type 1 phosphatase Dis2 to the cell tips (Alvarez-Tabares et al., 2007) and promoting Gef1 localization and Cdc42 activation at the site of cell growth by an unknown mechanism (Kokkoris et al., 2014).

Thus we tested the role of Gef1 phosphorylation by Orb6 kinase in the control of microtubule-dependent cell polarization. First, we analyzed the effect of microtubule depolymerization on the distribution of the CRIB-GFP marker. We found that treatment with microtubule inhibitor methyl benzimidazol-2-yl-carbamate (MBC) strongly increases CRIB-GFP asymmetry in gef1+ cells (Figure 5A, b and f) compared with DMSO-treated control cells (Figure 5A, a and e). Conversely, gef1S112A cells maintain a symmetrical CRIB-GFP distribution after MBC treatment (Figure 5A, d and h), similar to DMSO-treated control gef1S112A cells (Figure 5A, c and g), suggesting that loss of Orb6-mediated Gef1 phosphorylation suppresses the effects of microtubule depolymerization.

The microtubule-dependent Tea1–Tea4 complex functions as a targeting subunit for the type 1 phosphatase Dis2 at the cell tips, which has a role in local Gef1 recruitment and Cdc42 activation. Mutant cell cultures carrying the mutation tea4-v223a are unable to bind type 1 phosphatase and display decreased levels (30%) of bipolar cells (Supplemental Figure S4; Alvarez-Tabares et al., 2007; Kokkoris et al., 2014), showing monopolar cell growth (Figure S5B) and asymmetrical Gef1-3YFP localization (Figure S5B). We found that the gef1S112A mutation can suppress these phenotypes, promoting localization of Gef1-3YFP at the new end (Figure S5B) and bipolar growth activation in tea4-v223a cells (66% bipolar cells, similar to control tea+ cells; Figure S5B and Supplemental Figure S4, A and B). Collectively, our findings indicate that loss of Gef1 phosphorylation by Orb6 kinase rescues the bipolar growth activation defects associated with disruption of the microtubule cytoskeleton and loss of tip-localized type 1 phosphatase.

**Orb6-dependent control of Gef1 reveals a scaling relationship in the control of cell diameter**

*S. pombe* cells carrying the gef1S112A mutation display altered cellular dimensions (Figures 3Ad and 6A). Indeed, gef1S112A mutants display a cell diameter of 4.78 ± 0.2 μm, whereas control gef1+ cells display a cell diameter of 3.9 ± 0.2 μm (Figure 6B). To understand how Gef1 availability modulates the maintenance of cell diameter, we studied the functional interaction of Gef1 with other factors involved in diameter control. We found that moderately increasing the levels of Cdc42 GAP Rga4 by inducing its expression from the nmt1 promoter could fully rescue the wider diameter of gef1S112A mutants (3.13 ± 0.2 μm) compared with gef1S112A mutant cells alone (4.78 ± 0.2 μm; $p < 0.0001$) and closer to the diameter of control gef1+ cells (3.9 ± 0.2 μm; Figure 6, A and B, and Supplemental Figure S5, A and B). Stable induction of the thiamine-repressible nmt1 promoter was achieved by growth in thiamine-depleted minimal medium for 40 h. We found that nmt1-driven rga4 transcript levels were increased 30-fold over endogenous rga4 transcript levels (Supplemental Figure S5G), consistent with previous work addressing the strength of the nmt1 promoter (Maundrell, 1990). Conversely, deletion of rga4 leads to a synthetic phenotype in cells expressing Gef1S112A. gef1S112A rga4Δ cells are almost completely round, with a considerably increased cell diameter (6.05 ± 0.4 μm) compared with control cells or gef1S112A mutant cells (Figure 6, A and B; $p < 0.0001$).

To gain insight into how Gef1 and Rga4 cortical levels alter cell diameter by modulating the levels of active Cdc42 at each cell tip, we quantified the intensity of CRIB-GFP distribution at the plasma membrane in mutants expressing varying levels of Rga4 and Gef1. In wild-type cells, CRIB-GFP distribution at each tip follows a Gaussian (normal) distribution (Kelly and Nurse, 2011). CRIB-GFP distribution in gef1S112A cells also follows a Gaussian curve (Supplemental Figure S5C). We found that mutations altering the respective cortical levels of Gef1 and Rga4 reproducibly yielded Gaussian distributions with different spreads (Supplemental Figure S5D). Different Gaussian curves can be compared using the full-width at half-maximum (FWHM) value, which for Gaussian (normal) distributions is related to the SD σ (FWHM = $2\sigma \ln 2^{0.5} = 2.36\sigma$; Supplemental Figure SSD; Das et al., 2012). When we plotted the cell diameter as a function of the corresponding FWHM of CRIB-GFP distribution at the cell tips of different mutants (Figure 6D), we found that cell diameter was positively correlated with FWHM in an essentially linear manner (coefficient of determination = 0.988). Cells with a narrower CRIB-GFP distribution (lower FWHM; gef1+ nmt1-rga4 and gef1S112A nmt1-rga4) displayed a narrower cell diameter, whereas cells with a wider CRIB-GFP distribution (higher FWHM; gef1S112A and gef1S112A rga4Δ) displayed a wider cell diameter (Figure 6D).
Cell tip growth at the fission yeast cell tip depends on membrane expansion, mediated by the Cdc42-dependent exocyst function and actin cable assembly (Bendezu and Martin, 2011; Estravis et al., 2011). We speculated that establishment of cell diameter is directly dependent on the size of the growth area at the tip. In fission yeast cells, the shape of the growing tip can be quantified by using the Rcurve parameter, the radius of the curvature at the tip (Campas and Mahadevan, 2009; Estravis et al., 2011). We speculated that establishment of cell diameter is directly dependent on the size of the growth area at the tip. In fission yeast cells, the shape of the growing tip can be quantified by using the Rcurve parameter, the radius of the curvature at the tip (Campas and Mahadevan, 2009; Estravis et al., 2011).
FIGURE 6: Levels of the Cdc42 GEF Gef1 and Cdc42 GAP Rga4 modulate the distribution of active Cdc42 at the cell tips. (A) Fission yeast cell diameter increases with increasing cortical levels of Gef1 and decreasing levels of Rga4. gef1+ and gef1S112A mutants overexpressing Rga4 show the narrowest cell diameter, whereas gef1S112A rga4Δ mutant cells display a wide, almost spherical shape. Bar, 5 mm. (B) Quantification of cell diameter in mutants with varying levels of cortical Gef1 and Rga4. Statistical significance was validated when \( p < 0.05 \). The \( p \) values were determined using analysis of variance with SPSS statistics package 16.0 (IBM, New York, NY), followed by Tamhane’s post hoc test. (C) Activated Cdc42 (CRIB-GFP) localization at cell tips is distributed over a larger area in cells with increasing cortical levels of Gef1 and/or decreasing levels of Rga4. gef1+ and gef1S112A mutants overexpressing Rga4 show narrow CRIB-GFP distribution, whereas in gef1S112A rga4Δ mutant cells, CRIB-GFP distribution spreads over to the cell sides. (D) Positive correlation of cell diameter with FWHM of CRIB-GFP Gaussian distribution in mutants with varying levels of Rga4 and cortical Gef1. (E) Linear relationship between tip curvature (Rcurve) and cell diameter among mutants with varying levels of cortical Rga4 and Gef1. Rcurve corresponds to the radius of a circle traced along the curvature of the cell tip (Supplemental Figure S5E). (F) Relationship between tip curvature (Rcurve) and CRIB-GFP FWHM in mutants with varying levels of cortical Rga4 and Gef1.
Our observations reveal a cell-scale control mechanism in the regulation of cell polarization and cell morphology. Relative cortical levels of Gef1 and the Cdc42 GAP Rga4 dynamically regulate the Gaussian shape of active Cdc42 distribution, finely scaling up and down the area of cell growth and thus modulating the size of the cell tips, cell diameter, and overall cell proportions. Our findings indicate that by negatively regulating Gef1 function, Orb6 kinase globally affects the pattern of active Cdc42 distribution at the cell membrane, fostering the emergence of cell shape.

**DISCUSSION**

In this article, we describe a novel molecular mechanism in the control of cell morphology by Cdc42 activation. Our previous studies showed that active Cdc42 displays oscillatory dynamics at the cell tips (Das et al., 2012). Oscillations reveal the presence of positive and negative feedback controls in the system. Anticorrelated Cdc42 oscillations indicate the presence of competition for GTP-Cdc42 and/or its activators, leading to mutually exclusive activation of Cdc42 at the two tips (Das et al., 2012). The control system thus likely consists of Cdc42, Cdc42 regulators, and Cdc42 complex–binding sites on the membrane. Disruption of this control system alters active Cdc42 distribution at a cell-scale level at each tip and cell diameter (Das et al., 2012; Das and Verde, 2013).

Here we deciphered the mechanisms that limit Cdc42 activation by modulating the global level of active Cdc42. We showed previously that cell polarity is maintained through spatial control of active Cdc42 by the conserved NDR kinase Orb6 in a Cdc42 GEF Gef1-dependent manner (Das et al., 2009). Here we showed that Orb6 kinase negatively regulates cortical levels of Gef1, promoting competition for active Cdc42 at the cell tips. Increased active Cdc42 breaks the mutually exclusive activation at the two tips. Consistent with the idea that phosphorylation by Orb6 kinase alters Cdc42 GTPase dynamics, nonphosphorylatable gef1S112A mutants with increased cortical localization show decreased Cdc42 asymmetry (Figure 7A) and decreased anticorrelation of GTP-Cdc42 oscillations at the cell tips. Decreased Cdc42 asymmetry in gef1S112A mutants alters dependence of bipolar growth activation on a minimal cell size and accelerates the timing of bipolar growth activation (Figure 7C) as compared with wild-type cells (Figure 7B). Thus these findings reveal a mechanism that restrains the extent of global Cdc42 activation.

We show that Gef1 physically interacts with the 14-3-3 protein Rad24 in an Orb6-dependent manner. NDR/Orb6 generates a 14-3-3–binding site on Gef1 through phosphorylation, limiting Gef1

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**FIGURE 7:** Role of Orb6 kinase in the control of Cdc42 oscillatory dynamics, bipolar growth activation, and cell diameter. (A) Left, Orb6 kinase phosphorylates Cdc42 GEF Gef1 to promote Gef1 interaction with 14-3-3 protein Rad24. Gef1 sequestration by Rad24 limits Gef1 cortical levels at the cell tips and thus negatively regulates Cdc42 activation. The microtubule-dependent Tea4-PP1 landmark promotes Gef1 cortical localization and Cdc42 activation. Right, loss of Gef1 interaction with Rad24 leads to increased cortical Gef1 localization and Cdc42 activity, promoting bipolar growth activation irrespective of Tea4-PP1 function. (B–E). Regulation of Cdc42 GTPase activation by Orb6 kinase modulates the size of the area of cell growth and thus cell diameter and temporally controls cell growth in new areas of the cell (new-end take off). (B) Wild-type cells. (C) Increased cortical localization of Gef1 in gef1S112A mutants promotes Cdc42 activation, increasing cell width and promoting precocious bipolarity. (D) Loss of Rga4 in gef1S112A mutants widens the Gaussian distribution of Cdc42, further widening cell diameter. (E) Overexpression of Rga4 in gef1S112A mutants restores normal Cdc42 distribution at the cell tips, reducing cell diameter. The cooperative functions of Gef1 and Rga4 result in a “dynamic border” that controls cell polarization at each tip.
abundance at the tips (Figure 7A). Because rad24Δ results in increased cortical localization of Gef1, it is likely that phosphorylated Gef1 can localize to the cell cortex. By binding to phosphorylated Gef1, Rad24 sequestration in the cytoplasm represents one mechanism by which cells limit cortical Gef1 localization to the cell tips. Alternatively, it is also possible that Rad24 functions by displacing Gef1 at the tips. Future studies of Gef1 dynamics at the membrane will address these alternative possibilities. Rad24 was previously reported to be a major regulatory protein in fundamental cellular processes such as checkpoint control, cytokinesis, and transcriptional control (Ford et al., 1994; Forbes et al., 1998; Kitamura et al., 2001; Ozoe et al., 2002; Mishra et al., 2005; Chen et al., 2008). Rad24 also sequesters another S. pombe Rho GEF, Gef1, to prevent its localization to the nucleus, where Gef1 functions to protect cells against chronic replication stress (Muñoz et al., 2014). Here we describe a novel role for Rad24 in the spatial regulation of Cdc42 GTPase—sequestration of Gef1 to regulate the transition from monopolar to bipolar growth. Phosphorylation of Gef1 by Orb6 indicates that Orb6 behaves similarly to other NDR-type kinases including the LATS family of kinases involved in the control of cell cycle progression and apoptosis (Huang et al., 2005), through the generation of 14-3-3–binding sites (Chen et al., 2008; Hao et al., 2008). Our observations highlight a common modality of substrate regulation among these highly related kinases.

Further, our findings suggest that Orb6, in globally restricting Gef1 availability, has a role in promoting the dependence of Cdc42 activation on the microtubule cytoskeleton (Figure 7A). Microtubules naturally align along the cell main axis, have a role in maintaining the direction of polarized cell growth, and activate the new cell end by delivering the Tea1–Tea4 protein complex to the cell tips (Martin, 2009). Elegant work has shown that microtubules activate Cdc42 by recruiting Gef1 to the cell cortex in a Tea4-bound PP1–dependent manner (Alvarez-Tabares et al., 2007; Kokkoris et al., 2014). Our findings show that gef1s112A mutants bypass loss of Tea4–tethered PP1, suggesting a model in which Orb6 functions as a global inhibitor, opposing a microtubule-dependent local activator. Local activation, paired with global inhibition, forms a type of circuitry that is emerging as a unifying principle in cell polarization (Goehring and Grill, 2013). Future experiments will address whether the serine 112 in Gef1 is a direct target of the PP1. Alternatively, other Gef1-binding components (Rincon et al., 2007), Tea4–associated factors (Martin and Arkowitz, 2014), or other phosphatases (Bernal et al., 2012) could also participate in the modulation of Gef1 function.

The orb6 mutants are round (Verde et al., 1995, 1998), with dynamic CRIB-GFP patches forming throughout the cell cortex (Das et al., 2009). However, gef1s112A mutants show increased cell diameter but do not lose polarity. Thus Orb6 kinase likely regulates at least one more Cdc42 regulator in addition to Gef1. The Cdc42 GAP Rga4 is a likely candidate, as it physically (two-hybrid screen) and functionally interacts with Orb6 kinase (Das et al., 2007, 2009). Orb6 kinase possibly regulates both Gef1 and Rga4 to modulate Cdc42 activation, thus controlling cell dimensions and growth symmetry. Future work will address how Orb6 kinase regulates Rga4, which contains several potential Orb6 phosphorylation consensus sites that lie within putative 14-3-3–protein–binding sites.

Rga4 is enriched at the cell sides and was shown previously to have a role in the control of cell diameter (Das et al. 2007; Tatebe et al., 2008). In fission yeast, active Cdc42 GTPase distribution at each tip approximates a Gaussian distribution and resembles a gradient (Kelly and Nurse, 2011). Using mutants displaying different cortical levels of Gef1 and Rga4 (Figure 6, D and E), we show that the size of active Cdc42 Gaussian distribution has a positive correlation with cell diameter in a near-linear manner, likely because increasing tip-bound Cdc42 results in growth over a wider area. These observations indicate that distribution of Cdc42 activity is a major determinant of cell tip morphology both in terms of tip curvature (see later discussion) and cell diameter determination. Further, we show that GTP-Cdc42 self-organizes in a Gaussian manner at the cortex, even when the extent of Cdc42 activity varies considerably. Because Gaussian functions are solutions to the continuous diffusion equation, these findings suggest that diffusion-controlled processes regulate active Cdc42 GTPase distributions in all these mutants.

Collectively these observations suggest that the opposing functions of Gef1 and Rga4 continuously modulate the cortical gradient of activated Cdc42, resulting in a “dynamic border” that controls cell polarization at each tip (Figure 7, D and E). This mechanism, in cooperation with other mechanisms of control, such as the negative feedback promoted by the Cdc42–dependent Pak1 kinase, which also limit the extent of Cdc42 activation, promotes Cdc42 oscillatory dynamics (Das et al., 2012). Consistently, orb6-25 and pak1/orb2-34 temperature-sensitive mutants display synthetic lethal interactions (Verde et al., 1995, 1998).

Our results reveal another feature of size scaling at the cell tips; we show that increased levels of GTP-Cdc42 lead to cell tips with a wider curvature. Recent theoretical models of cell shape addressed scaling laws in the control of tip morphogenesis. It was proposed that secretion of cell wall material and differential cell wall viscosity modulate tip pointedness, promoting a range of possible cell diameters and tip shapes for which the radius of tip curvature and the radius of the cell body differ (Campas and Mahadevan, 2009; Drake and Vavylonis, 2013). However, we find experimentally that in fission yeast, tip radius and cell diameter are directly proportional upon varying Cdc42 activity, indicating a scaling relationship between tip curvature and cell width. This effect may be due to the dependence of secretion on Cdc42 activity (Estravis et al., 2011), which likely also modulates cell wall maturation via delivery of cell wall biosynthetic enzymes (Estravis et al., 2012). In addition, it is possible that unknown feedback mechanisms, dependent on physical principles such as cell wall viscosity or turgor pressure, may have a role in modulating Cdc42 activity.

Finally, we propose that in fission yeast, by modulating Gef1 availability, the conserved NDR kinase Orb6 establishes the overall scale of cellular dimensions and the number of growing areas. We find that Gef1 is a homologue of the mammalian Cdc42 GEF TUBA/DNMBP, a protein implicated in epithelial cell polarization and associated with late-onset Alzheimer’s disease (Kuwano et al., 2006; Bettens et al., 2009; Bryant et al., 2010; Kovacs et al., 2011; Zuo et al., 2011). In mammalian cells, NDR kinases are part of the Hippo tumor suppressor pathway (Hergovich and Hemmings, 2009; Cornils et al., 2011) and have been implicated in neuronal development (Vichalkovski et al., 2008; Emoto, 2011). Because of the conservation of these regulatory modules, it is likely that our findings will have implications for the understanding of cell polarization and other processes in higher eukaryotes.

MATERIALS AND METHODS
Strains and cell culture
S. pombe strains used in this study are listed in Supplemental Table S1. All strains used in this study are isogenic to the original strain 972. Fission yeast cells were cultured in yeast extract (YE) medium or in Edinburgh minimal medium (EMM) plus required supplements. Genetic manipulations and analyses were carried out using standard

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gef1::ura4 was then transformed into PPG2601 (using a QuikChange Kit [Stratagene, La Jolla, CA]). The construct Gef1 promoter (from position –650 to position –1) was cloned along with the full-length Gef1 gene into the pFA6-3YFP plasmid. gef1S112A mutants were generated by site-directed mutagenesis using a QuikChange Kit (Stratagene, La Jolla, CA). The construct was then transformed into PPG2601 (gef1::ura4+ leu1-32 ura4-D18) cells, selecting for G418-resistant colonies. The resulting strain contained one functional copy of gef1S112A fused to 3YFP generated by gef1::ura4+ sequence. Deletion mutants of gef1 were generated by PCR amplification of different regions of Gef1 open reading frame with the Gef1 promoter (from position –650 to position –1), cloned into the pFA6-3YFP plasmid, and transformed in PPG2601.

In vitro kinase assays and mass spectrometry
Orb6 kinase assay was performed as described in Wiley et al. (2003). Myc-tagged Mob2–expressing cells (PV558) and control cells expressing orb6-as2 mutant were grown in YE to mid log phase at 32°C. Cells were broken using Savant FastPrep FP120 bead beater in HB buffer (25 mm 3-(N-morpholinol)propanesulfonic acid, pH 7.2, 60 mM β-glycerophosphate, 15 mM p-nitrophenyl phosphate, 15 mM MgCl2, 15 mM ethylene glycol tetracetic acid, 1 mM dithiothreitol, 0.1 M sodium vanadate, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride plus protease inhibitors [complete EDTA-free protease inhibitor cocktail tablets; Roche Applied Science]). Protein G Dynabeads (Invitrogen, Waltham, MA) magnetic beads bound to rabbit anti-Myc antibodies (Santa Cruz Biotechnology) were incubated with (1 mg/ml) protein extracts from orb6-as2 cells and Myc-tagged Mob2–expressing cells for 1 h and then washed twice with HB buffer and once with kinase buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 1 mM MnCl2). The beads were then resuspended in 25 μl of kinase buffer containing 10 μCi of [γ-32P]ATP (6000 Ci/mmol), 20 μM ATP, and 5 μl of bacterially expressed hexahistidine-tagged N-terminal Gef1. The kinase reaction was incubated for 20 min at 30°C and terminated by added Laemml buffer, and boiling proteins were then separated on a SDS–polyacrylamide gel.

Mass spectrometry analysis
The in vitro kinase assay was performed with Myc-tagged Mob2–associated Orb6 kinase using bacterially expressed hexahistidine-tagged N-terminal Gef1 as described, with the following exceptions. The samples were split in two; one-half was treated with radioactive [γ-32P]ATP, and the other half was treated with nonradioactive ATP for the kinase assay. The radioactive sample was then loaded on SDS–PAGE gels to detect Gef1 phosphorylation. The nonradioactive sample was trichloroacetic acid (TCA) precipitated as follows. TCA, 100%, was added to the sample to get a final concentration of 20% TCA in ice. The reaction was incubated in ice overnight in a cold room. The reaction was then centrifuged for 30 min at 4°C at 13,000 rpm. The supernatant was discarded by aspiration with only 5–10 μl remaining in the tube. The reaction was then washed with 500 μl of ice-cold acetone twice, with 10-min centrifugation after each wash. The protein digest was pressure loaded onto a fused-silica 100-μm–inner diameter capillary with a 5-μm pulled tip packed with 10 cm of 3-μm Aqua C18 material (Phenomenex, Ventura, CA) placed inline with an Agilent 1100 quaternary HPLC (Palo Alto, CA). The buffer solutions used were 5% acetonitrile/0.1% formic acid (buffer A) and 80% acetonitrile/0.1% formic acid (buffer B). As peptides eluted from the microcapillary column, they were electro-sprayed directly into an LTQ two-dimensional ion trap mass spectrometer (ThermoFinnigan, Palo Alto, CA) with the application of a distal 2.4-kV spray voltage. A cycle of one full-scan mass spectrum (400–1400 m/z) followed by eight data-dependent tandem mass spectrometry (MS/MS) spectra at a 35% normalized collision energy was repeated continuously throughout each step of the multidimensional separation. MS scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Scientific, Waltham, MA).
Analysis of tandem mass spectra

Tandem mass spectra were analyzed using the following software analysis protocol. Poor-quality spectra were removed from the data set using an automated spectral quality assessment algorithm (Bern et al., 2004). Tandem mass spectra remaining after filtering were searched using the SEQUEST algorithm (Eng et al., 1994) against the Schizosaccharomyces pombe protein sequence database concatenated to a decoy database in which the sequence for each entry in the original database was reversed (Peng et al., 2003). All searches were parallelized and performed on a Beowulf computer cluster consisting of one hundred 1.2-GHz Athlon CPUs (Sadygov, 2002). The MS/MS spectra were searched to consider phosphorylation modifications of +80 on STY. SEQUEST results were assembled and filtered using the DTASelect, version 2.0, program (Tabb et al., 2002; Cocciora et al., 2007). DTASelect 2.0 uses a linear discriminant analysis to dynamically set XCorr and DeltaCN thresholds for the entire data set to achieve a user-specified false-positive rate (5% in this analysis). The false-positive rates are estimated by the program from the number and quality of spectral matches to the decoy database.

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