The RHO1-specific GTPase-activating Protein LRG1 Regulates Polar Tip Growth in Parallel to Ndr Kinase Signaling in Neurospora

Nico Vogt*† and Stephan Seiler*†

*Institut für Mikrobiologie und Genetik, Abteilung Molekulare Mikrobiologie; and †Deutsche Forschungsgemeinschaft Research Center of Molecular Physiology of the Brain (CMPB), Universität Göttingen, D-37077 Göttingen, Germany

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Regulation of Rho GTPase signaling is critical for cell shape determination and polarity. Here, we investigated the role of LRG1, a novel member of the GTPase-activating proteins (GAPs) of Neurospora crassa. LRG1 is essential for apical tip extension and to restrict excessive branch formation in subapical regions of the hypha and is involved in determining the size of the hyphal compartments. LRG1 localizes to hyphal tips and sites of septation via its three LIM domains. The accumulation of LRG1 as an apical cap is dependent on a functional actin cytoskeleton and active growth, and is influenced by the opposing microtubule-dependent motor proteins dynein and kinesin-1. Genetic evidence and in vitro GTPase assays identify LRG1 as a RHO1-specific GAP affecting several output pathways of RHO1, based on hyposensitivity to the glucan inhibitor caspofungin, synthetic lethality with a hyperactive GTPase, and hypersensitivity to latrunculin A. The morphological defects of lrg-1 are highly reminiscent to the Ndr kinase/RAM pathway mutants cot-1 and pod-6, and genetic evidence suggests that RHO1/LRG1 function in parallel with COT1 in coordinating apical tip growth.

INTRODUCTION

Polarized growth is a multifactorial property that is coordinated by numerous signaling pathways, and monomeric GTPases of the Ras super family have been identified as key regulators of cell polarity (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005; Bustelo et al., 2007; Park and Bi, 2007). They act as molecular switches that cycle between an active GDP–bound and an inactive GTP–bound form. Transition between these two forms is achieved through GTPase-activating proteins (GAPs) leading to the inactive form and GDP–GTP–exchange factors (GEFs) that activate the small G-protein. Originally, GTPases of the Rho subfamily were described as key regulators of the actin cytoskeleton, but now it has become obvious that they influence many cellular processes including cytoskeletal organization, vesicle transport, and transcriptional regulation. The position of small G-proteins at the bottleneck of signal transduction pathways explains the many defects seen when these GTPases are misregulated.

A challenging complication in determining the specific functions of small G-proteins is the fact that the number of GAPs and GEFs is significantly larger than the number of GTPases that they regulate. For example, the D. melanogaster genome contains only six Rho proteins, but ~20 GEFs and more than 20 GAPs (Adams et al., 2000), indicating that understanding the specific functions of these regulators will be the key for deciphering the temporal and spatial activity of the switches. Although considerable progress has been made in understanding the activation of small GTPases through their GEFs (Gulli and Peter, 2001; Schmidt and Hall, 2002; Garcia et al., 2006), full activation of a specific GTPase requires not only the coordination of the “on” switch, but also the shutting between both the active and inactive states of the GTPase is essential for full signaling activity of the small G-protein (Vanni et al., 2005; Barale et al., 2006). Nevertheless, little is known about how the GAPs are regulated in a spatial and temporal manner. Interestingly, the number of GTPases present in the available fungal and animal genomes is exceeding those of the GEFs (Goffeau et al., 1996; Adams et al., 2000; Borkovich et al., 2004; Barale et al., 2006), indicating that fine-tuning of the “off-switch” is of high importance for providing the necessary specificity for the Rho module.

Apical tip extension is the hallmark of filamentous fungi, and fungal hyphae share with neurons and pollen tubes the distinction of being among the most highly polarized cells in biology (Palanivelu and Preuss, 2000; Chilton, 2006; Harris, 2006), thus making them attractive models for the analysis of fundamental mechanisms underlying cellular polarity. Nevertheless, the molecular understanding of fungal morphogenesis is still a major challenge. Phylogenetic analyses and the comparison of Saccharomyces cerevisiae morphogenetic data with the limited results from various filamentous ascom and basidiomycetes have established that a core set of “polarity factors” is conserved between unicellular and filamentous fungi (reviewed in Wendland, 2001; Borkovich et al., 2004; Harris, 2006). Thus, the accumulated knowledge of baker’s yeast is serving as an invaluable source for comparative morphogenetic studies, but it is becoming increasingly evident that subtle differences in the wiring of these con-
served components and the presence of additional proteins that are absent in unicellular fungi result in dramatically different morphogenetic outcomes ranging from budding to filamentous growth (Boyce et al., 2003, 2005; Rottmann et al., 2003; Seiler and Plamann, 2003; Li et al., 2006; Malavazi et al., 2006).

Current fungal research is focusing on the description of the various Rho proteins and the analysis of the interplay between the different modules (Wendland and Filippsen, 2000, 2001; Boyce et al., 2003, 2005; Chen and Dickman, 2004; Levelek et al., 2004; Rasmussen and Glass, 2005, 2007; Mahlert et al., 2006). Various studies have identified Rh1 as one key regulator of hyphal growth and polarity. Aspergillus fumigatus Rh1 has been described as part of the B1,3-glucan synthase complex that localizes to zones of active growth at the hyphal apex (Beauvais et al., 2001). A similar role in maintaining cell wall integrity was suggested for Rh1 of Ashbya gossypii, because deletion mutants showed reduced filamentous growth and high rates of cell lysis (Wendland and Filippsen, 2001). Aspergillus nidulans RhA has been implicated in polar growth, branching, and cell wall synthesis (Guest et al., 2004). Budding yeast Rhop1 as the best characterized representative of the Rh1 family has multiple functions in regulating the two main structural features of the fungal cell (reviewed in Levin, 2005; Park and Bi, 2007). The organization of the actin cytoskeleton is controlled through the interaction of the activated GTPase with the polarisome component Bni1p, whereas maintenance of the cell wall integrity is achieved via two independent mechanisms. Rhop1 activates cell wall synthesis through the direct stimulation of the enzyme glucan synthase, which catalyzes the polymerization of B1,3-glucan. In addition, it activates the mitogen-activated protein (MAP) kinase pathway that monitors the cell wall integrity through its activation of protein kinase C thereby coordinating the transcription of several cell wall–specific enzymes.

To identify the critical components that contribute to polarized growth, we developed a large-scale genetic screen for the isolation of conditional mutants defective in polar and directed growth in the Neurospora crassa (Seiler and Plamann, 2003) and have identified the Ndr kinase COT1 and its upstream regulator kinase POD6 as required for polarized morphogenesis. Here, we describe the characterization of a LIM and Rho-GAP domain–containing protein (therefore named LRG1) that acts as a RHO1-specific GAP and provide genetic evidence that LRG1 functions in parallel with COT1 in coordinating polar tip growth.

Materials and Methods

Strains, Media, and Growth Conditions

N. crassa strains used in this study are summarized in the Table 1. General genetic procedures and media used in the handling of N. crassa have been described (Roon and Deberardinis, 1970) or are available through the fungal Genetic Stock Center (www.fgsc.net). Growth tests to determine the sensitivity of mutant strains in comparison to wild type against cytoketal, cell wall, and protein kinase C (PKC) inhibitors were performed on minimal medium supplemented with 0.5% sodium acetate to enhance colony formation and allow a better comparison of the different strains on a single plate. The hygromycin B and nourseothricin concentrations were adjusted to 200 and 30 μg/ml, respectively, to select for transformants. Homologous recombination in Neurospora hyphal strain HP1 containing the n serval nucleus were verified by phenotypic analysis of transformants grown on 400 μM 3-fluorophenylalanine (fpa) and 200 μg/ml histidine or 5 μg/ml benomyl and 200 μg/ml pantothenic acid and by the complementation of the growth defect of potential Δlrgr1 strains with a 6-kb genomic SacII fragment containing Δlrgr1 (pNV16).

Plasmid Construction

Plasmid construction

Primer sequences are summarized in the Supplementary Table S1. The Δlrgr1 deletion cassette in pNV46 was obtained by plasmid gap repair in S. cerevisiae (Orr-Weaver and Szostak, 1983). pRS416 was linearized with XbaI and Xhol, the Nat° cassette was amplified by PCR with primers NV_gap1 and NV_nat5 and digested in template (Seiler and Plamann, 2003) and the 3' portions of the deletion cassette in yeast. To generate the GAP and LIM domain deletion constructs, a 6-kb genomic SacII fragment containing Δlrgr1 coding region and 1.5- and 1-kb 5' and 3' regions, respectively, was inserted in pBluescript SK+ (Strategene, Amsterdam, Netherlands) to obtain pNV16. The hygromycin cassette, amplified with the primers Hyg5′/Xba and Hyg3′/Xba and plasmid pMPl6 as template, was inserted into the unique XbaI site. LRG1Δlrgr1 was generated by deleting the C-terminal domain of Δlrgr1 in pNV16 with Apal and by inserting the which was amplified with the primers NV_myc3 with pYM6 (Knop et al., 2002). The LRG1Δlrgr1/Δlrgr1 strain was rescued by transformation with a mating type switch. The Δlrgr1 deletion in the genome of LRG1Δlrgr1 and LRG1Δlrgr1/Δlrgr1 strains was confirmed by PCR analysis of genomic DNA with specific primers. The primers NV_LRG20 and NV_LRG21, and the hygromycin resistance cassette introduced via the EcoRI site of pBluescript SK+ (Stratagene, La Jolla, CA) were amplified with primers NV_GAPmut_r, and the hygromycin resistance cassette introduced into the unique NotI site of the vector. The R847L point mutation in the GAP domain was generated with the QuickChange Site-Directed Mutagenesis Kit (Strategene) according to manufacturer’s instruction using the oligonucleotides NV_LIM1mut_f, NV_LIM1mut_r, NV_LIM4mut2_f, NV_LIM4mut2_r, NV_LIM2mut_f, NV_LIM2mut_r, NV_LIM3mut2_f, NV_LIM3mut2_r, NV_LIM3mut3_f, NV_LIM3mut3_r, NV_LIM3mut2_r, NV_LIM3mut3_r. The zinc-finger coordinating cysteines and histidines were substituted by amino acids that lack the zinc-binding capability (LIM1: C121S, C124S, C98L, C101S; LIM2: H185C, C188G; LIM3: C495S, C527S). The primers NV_LIM1mut_f, NV_LIM1mut_r, NV_LIM4mut2_f, NV_LIM4mut2_r, NV_LIM2mut_f, NV_LIM2mut_r, NV_LIM3mut2_f, NV_LIM3mut2_r, NV_LIM3mut3_f, NV_LIM3mut3_r were designed in the QuickChange Site-Directed Mutagenesis Kit (Strategene) according to manufacturer’s instruction using the oligonucleotides NV_LIM1mut_f, NV_LIM1mut_r, NV_LIM4mut2_f, NV_LIM4mut2_r, NV_LIM2mut_f, NV_LIM2mut_r, NV_LIM3mut2_f, NV_LIM3mut2_r, NV_LIM3mut3_f, NV_LIM3mut3_r. The zinc-finger coordinating cysteines and histidines were substituted by amino acids that lack the zinc-binding capability (LIM1: C121S, C124S, C98L, C101S; LIM2: H185C, C188G; LIM3: C495S, C527S). The primers NV_LIM1mut_f, NV_LIM1mut_r, NV_LIM4mut2_f, NV_LIM4mut2_r, NV_LIM2mut_f, NV_LIM2mut_r, NV_LIM3mut2_f, NV_LIM3mut2_r, NV_LIM3mut3_f, NV_LIM3mut3_r were designed in the QuickChange Site-Directed Mutagenesis Kit (Strategene) according to manufacturer’s instruction using the oligonucleotides NV_LIM1mut_f, NV_LIM1mut_r, NV_LIM4mut2_f, NV_LIM4mut2_r, NV_LIM2mut_f, NV_LIM2mut_r, NV_LIM3mut2_f, NV_LIM3mut2_r, NV_LIM3mut3_f, NV_LIM3mut3_r. The zinc-finger coordinating cysteines and histidines were substituted by amino acids that lack the zinc-binding capability (LIM1: C121S, C124S, C98L, C101S; LIM2: H185C, C188G; LIM3: C495S, C527S). The primers NV_LIM1mut_f, NV_LIM1mut_r, NV_LIM4mut2_f, NV_LIM4mut2_r, NV_LIM2mut_f, NV_LIM2mut_r, NV_LIM3mut2_f, NV_LIM3mut2_r, NV_LIM3mut3_f, NV_LIM3mut3_r were designed in the QuickChange Site-Directed Mutagenesis Kit (Strategene) according to manufacturer’s instruction using the oligonucleotides NV_LIM1mut_f, NV_LIM1mut_r, NV_LIM4mut2_f, NV_LIM4mut2_r, NV_LIM2mut_f, NV_LIM2mut_r, NV_LIM3mut2_f, NV_LIM3mut2_r, NV_LIM3mut3_f, NV_LIM3mut3_r.
Table 1. Neurospora crassa strains used in this study

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primers NV<sub>lrg14</sub> and NV<sub>lrg18</sub> and introduced via SalI/XhoI and NotI
adapted from Minke et al. (1999). Conidia were germinated on a small piece
of GN-6 cellulose filter (Gelman Sciences,) placed on the surface of an agar
plate. Filters were plunge-frozen in liquid propane and transferred to fixative
(3% formaldehyde in 100% ethanol precooled to
80°C). Samples were main-

Microscopy

Immunolocalization for N. crassa hyphae was conducted following a protocol adapted from Minke et al. (1999). Conidia were germinated on a small piece of GN-6 cellulose filter (Gelman Sciences,) placed on the surface of an agar plate. Filters were plunged-frozen in liquid propane and transferred to fixative (3% formaldehyde in 100% ethanol precooled to 80°C). Samples were maintained at –80°C for at least 2 d and then slowly transferred to room temperature (2 h at 20°C, 2 h at 4°C). Filters were rehydrated in a series of ethanol: buffer (100 mM phosphate, pH 7.0) solutions starting at a ratio of 90:10 and ending at 90:0. The cell wall was digested by incubating the filters for 0.5–8 min in 2 mg/ml lyzing enzymes from Trichoderma harzianum (Sigma, Munich, Germany) in 100 mM potassium citrate (pH 6.0, 20 mM EGTA, 5% BSA). To block nonspecific binding of antibody, the filters were incubated for 1 h in 5% BSA. Samples were immersed in the primary antibody for at least 8 h, washed several times in phosphate buffer, incubated in the secondary antibody for 2 h, and visualized using standard rhodamine and FITC filter sets. For immuno-
localization studies of GFP::LRG1 and GFP::LRG1*, an AxioObserver.Z1 mi-
croscope equipped with an ApoTome unit, an AxioCam MRm r3.0 CCD camera and X-Cite (EXFO) illumination source was used with AxioVision Software 4.6. For confocal imaging an Axiovert 100M microscope with the confocal module LSM 510 and LSM 510-Software was used (all from Zeiss). To determine the kinetics of septum formation, DIC imaging of a hyphal segment was randomly started, and the fluorescence images were manually recorded at time points when the LRG::GFP localization had visibly changed to reduce photobleaching. The absolute time points of the GFP images were used for the calculation of septation kinetics. Low magnification documentation of fungal hyphae or colonies was performed with a SZX12 stereomicroscope (Olympus, Tokyo, Japan) and a PS30 video camera (Kappa, Gleichen, Germany).

Enzymatic Assays

The RHO GTPases were purified as glutathione S-transferase (GST) fusion proteins. Transformed Rosetta2(DE3) cells (Novagen, Schwabach, Germany) were grown at 20°C and induced for 12 h with 0.1 M IPTG. Cell extracts generated by sonication in lysing buffer (50 mM Tris, pH 7.5, 10% saccharose, 5 mM MgCl<sub>2</sub>, 1 mM PMF, 0.008% β-mercaptoethanol, and 0.02% NP40), were bound to GSH Sepharose (Amersham, Buckinghamshire, United Kingdom), washed (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM PMF, 0.008% β-mercaptoethanol, 0.02% NP40) and eluted (50 mM Tris, pH 8.0, 250 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM DTT, 20 mM glutathione reduced, and 0.02% NP40). GSTase assays were performed as described (Gibbs et al., 1988). Pre-equilibration of the RHO proteins was for 15 min at
LRG1 is a RHO1-specific GAP in *Neurospora*

**RESULTS**

**LRG1 Is Essential for Hyphal Tip Extension**

The phenotypic analysis of *lrng-1(12-20)* indicated no major differences in hyphal morphology or branching frequency compared with wild type when the strains were grown at permissive temperature (25°C; Figure 1, A and B). However, within 30 min of transferring *lrng-1(12-20)* to 37°C, we observed a rapid cessation of tip extension. In contrast to the wild-type apex, which is a dome-shaped structure, the tips of *lrng-1(12-20)* generated a characteristic pointed, needle-like shape. This stop of tip extension was accompanied by the appearance of numerous subapical needle-shaped branches of ≤10 μm in length that also stopped growth with a pointed tip. After prolonged incubation at restrictive temperature, the new branches as well as the primary hyphae began to swell up in a bulbous and apolar manner. Transfer of such a culture back to permissive temperature resulted in growing tips having normal growth rates, diameter, and morphology within 30 min. Germination of *lrng-1(12-20)* at restrictive temperature resulted in the formation of compact colonies with multiple ≤10-μm-long germ tubes with pointed tips.

Chitin is the primary component of fungal septae and of the inner layers of the hyphal cell wall and is therefore accessible to the Calcofluor White staining, primarily at the hyphal tips and at septae. At permissive temperature, the chitin distribution patterns were observed. Within 30 min at 37°C, *lrng-1(12-20)* showed extensive label in a patchy, subapical manner throughout the hyphae at positions of newly emerging branches, indicating excessive chitin deposition at sites.

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**Figure 1.** LRG1 is essential for hyphal tip extension. (A) Wild type was grown on minimal media plates at 25°C (left, DIC image; right, Calcofluor White staining). Bar, 40 μm. (B) *lrng-1(12-20)* was grown on minimal media plates at 25°C and shifted for the indicated times to restrictive temperature to illustrate the cessation of tip extension with pointed, needle-like tips and the progressive hyperbranching of the mutant (phase-contrast images, top panel). Increased septation and abnormal chitin distribution were monitored by labeling with Calcofluor White (bottom panel). Bar, 40 μm. (C) Growth of the heterokaryotic *lrng-1* deletion strain *lrng-1* on minimal medium, medium containing fpa and histidine and medium containing benomyl and pantothenic acid (which forces the knockout nucleus to predominate), resulting in morphological defects identical to *lrng-1(12-20)* germinated at restrictive temperature. Bar, 7.5 μm.
of aberrant growth. In contrast, the high Calcofluor White stain at the primary tips observed in hyphae grown at the permissive temperature is nearly absent when the strains are cultured at the restrictive temperature, indicating decreased chitin synthesis at these nonelongating tips. Furthermore, we observed increased septation of lrg-1(12-20) compared with wild type grown at permissive temperature, which is further increased after shifting cells to restrictive conditions. The length of hyphal compartments at 25°C were 53.7 ± 21.0 and 148 ± 58.0 μm for lrg-1(12-20) and wild type, respectively (n = 50). After the transfer of lrg-1(12-20) to 37°C for 2.5 h the compartment length was further reduced to 11.3 ± 4.3 μm (n = 50), whereas the compartment length of wild type remained unchanged. The hyphal diameter of wild type and lrg-1(12-20) were similar and did not change during the shift experiment (8.2 ± 0.8 and 8.4 ± 1.6 μm, respectively, after 2.5 h at 37°C; n = 20).

To examine the phenotype of a lrg-1 deletion strain, we constructed a null mutant by using the sheltered disruption method (Nargang et al., 1995), which takes advantage of the fact that N. crassa is a multinucleated cell. The lrg-1 gene was deleted via homologous recombination in the heterokaryotic strain HP1, thereby allowing the generation of mutants in genes that are essential or important for growth. The resulting mutant harbors two kinds of nuclei, one with a null allele of lrg-1, and one with a wild-type copy. These nuclei contain selectable markers that allowed a shift in the nuclear ratio within the heterokaryotic cells. Growth on media containing Ipa and histidine favored the propagation of the wild-type nucleus. In contrast, growth of heterokaryotic cells on media containing benomyl and pantothenic acid forced the knockout nucleus to predominate and thus led to the depletion of LRG1 and morphological defects that were indistinguishable from lrg-1(12-20) germinated at restrictive temperatures (Figure 1C). These results characterize lrg-1(12-20) as a conditional loss-of-function allele of lrg-1 and confirm an essential role of LRG1 during the apical extension of the hyphal tip and in controlling the number and position of subapical branches.

LRG1 Contains Three LIM Domains and a GAP Domain That Is Essential for Growth and Septation

Examination of the 1279 amino acid (aa) encoding LRG1 sequence revealed two interesting features (Figure 2A). The N-terminal region from aa 1–533 is cysteine- and histidine-rich and can be arranged into three tandem zinc-finger-containing structures called LIM domains that act as versatile protein-protein interaction motifs (Schaller, 2001; Brown and Turner, 2004). Furthermore, a RHO-GAP domain was found in the C-terminal part of LRG1 (aa 791-1279). Sequence comparisons indicated that highly homologous proteins containing this domain architecture are present in all available fungal genomes. Apart from the fungal LRG1 relatives, the LIM domains of LRG1 are most closely related to the focal adhesion organizing protein paxillin (E-value of 2e−28 for Drosophila pseudobscura paxillin vs. E = 1e−17 for mouse leupaxin, a more distantly related member of the paxillin superfamily, and E = 1e−14 for human actin-binding LIM protein 1 as the closest non-paxillin-related LIM domain-containing hit).

Sequencing of lrg-1(12-20) revealed a single amino acid substitution of the conserved tyrosine 926 (TAC) in the GAP domain by histidine (CAC), suggesting that the GAP domain is essential for the cellular function of LRG1. To analyze the function of the two LRG1 domains, we generated and expressed constructs lacking either the three LIM (LRG781–1279) or the GAP (LRG1–847) motifs in lrg-1(12-20), and determined that the two individual domains were unable to complement the tip extension defect of lrg-1(12-20) (Figure 2B). The importance of the GAP domain for hyphal growth was further supported by a construct, in which the conserved lysine 910 in the full-length protein was substituted with alanine. This mutation has been shown to result in a nonfunctional GAP due to loss of binding to the corresponding Rho (Li et al., 1997). When we expressed this construct in lrg-1(12-20) or in Δlrg-1, no complementation of the mutants defects were detected (Figure 2B). To confirm that the GAP activity of LRG1 is essential for its function, we generated an allele, in which the conserved catalytic arginine residue 847 of the GAP domain was substituted by a lysine. This construct was expressed in lrg-1(12-20), but was not sufficient for complementation (Figure 2B), thus providing strong evidence for the importance of the enzymatic activity for the function of LRG1.

An important function of the N-terminal part of LRG1 containing the three LIM domains was indicated by the failure of LRG1781–1279 to complement lrg-1(12-20). Therefore, we generated a series of lrg-1 alleles, in which four of the eight metal ion-coordinating cysteines or histidines of each of the three LIM domains were substituted with serines or alanines. These residues have been shown to mimic the behavior of cysteines, but lack their zinc-coordinating activity, which has been shown to be essential for the function of LIM domains (Schmeichel and Beckerle, 1997; Bombarda et al., 2002). Surprisingly, when we tested the three constructs that each was defective in one of the three LIM domains, we found that they did lead to full phenotypic complementation of lrg-1(12-20). Also, when we combined the three mutated LIM domains by generating LRG*, we did not observe abnormal growth of the lrg-1(12-20) (Figure 2B) or of homocaryotic Δlrg-1 transformants (data not shown) and measured overall radial growth rates on agar plates, which were comparable to wild type (LRG1: 1.1 ± 0.1 mm/h; LRG1*: 1.0 ± 0.4 mm/h; n = 3). However, we noted a higher variability of extension rates of individual hyphal tips of LRG1* grown on microscopic slides (7.2 ± 6.2 μm/min; n = 50) in comparison to LRG1 (6.3 ± 4.3 μm/min; n = 50), suggesting that the dysfunctional LIM domains impair proper tip extension. Nevertheless, the placement of septa was not altered in LRG1*. The distance of the hyphal tip to the first septum was 224.0 ± 41.9 μm in LRG1 and 225.4 ± 25.8 μm for LRG1* (n = 30), whereas the distance between subapical septae was 84.9 ± 30.9 μm in LRG1 compared with 92.5 ± 32.4 μm in LRG1* (n = 100).

The LIM Domains Are Required for Localizing LRG1 to Sites of Growth

To determine the cellular distribution of LRG1, we generated an N-terminal myc™-tagged version of lrg-1, which complemented lrg-1(12-20). Immunolocalization experiments using anti-MYC antibodies revealed a punctate distribution throughout the cell, which was enriched at hyphal tips (Figure 3A). This localization was in line with a function of LRG1 at growing tips and was also observed, when we used antisera generated against LRG1 (data not shown). Next, we characterized the localization pattern of MYC™:LRG11–847 and LRG781–1279:MYC™ in the wild-type background. Although LRG781–1279:MYC™ aberrantly localized in a cortical manner throughout the whole hypha and along septae, the MYC™:LRG11–847 distribution was similar to localization of LRG1, suggesting that the N-terminal part of LRG1 is involved in the localization of the GAP domain.

For a better resolution of the dynamics of LRG1, we generated N-terminal GFP fusion constructs with LRG1 and
with LRG1* (containing the three mutated LIM domains). Both constructs were introduced into homocaryotic Δlrg-1 and complemented the growth defect of the deletion strain. ApoTome-based fluorescence microscopy confirmed the apical vesicular-reticulate localization of GFP::LRG1, but resulted in a more diffuse localization of GFP::LRG1* lacking the prominent streaks (Figure 3B). In addition to this tip-enriched vesicular–reticulate localization, we frequently observed a GFP::LRG1 cap along the apical cortex and strong staining around the septal pore (Figure 3C). In contrast, GFP::LRG1* was distributed in a more diffuse manner throughout the hypha with only a weak accumulation at the hyphal apex and no localization at septae. Thus, both the immunolocalization data and the GFP-fusion proteins indicated a function of the LIM domains in localizing the GAP domain to sites of active growth in the apical region and along septae.

Confocal microscopy was used to characterize the dynamics of GFP::LRG1 in more detail. We first asked, if the localization of the apical cap is growth-dependent and analyzed 100 randomly chosen tips of the GFP::LRG1 expressing strain (Figure 4A). At a growth rate of ≥0.2 μm/s, 70% displayed an apical cap, whereas 2% did not. In contrast, only 10% of the hyphae growing at rates of <0.2 μm/s and displayed an apical cap, whereas 18% did not. Thus, the localization of GFP::LRG1 as a cap-like structure at the hyphal tip was dependent on active growth. When we quantified the localization of GFP::LRG1*, we observed the opposite behavior. Only 13% fast-growing and 2% slow-growing tips displayed a cap, whereas 62 and 23%, respectively, did not show an apical cap of GFP::LRG1, confirming that the LIM domains are responsible for localizing LRG1 to the growing hyphal apex in addition to their importance in targeting LRG1 to sites of septation.

During this analysis, we noted that the size of the apical cap varied with the growth rate. GFP::LRG1 accumulated in slow growing hyphae primarily along the central apical cortex, whereas in fast growing tips, an extended cap structure was visible, with the highest GFP intensity frequently accumulating as a subapical ring ~2 μm behind the apex

**Figure 2.** The N-terminal region and the C-terminal Rho-GAP domain are both required for LRG1’s cellular function. (A) Genomic organization of lrg-1 locus. The four exons are represented in white, the three introns in black. The lrg-1 gene encodes for a protein with three LIM domains (brown) in the N-terminal half and one Rho-GAP domain (blue) in the C-terminal part of the protein. Green arrows represent expression constructs under the control of the endogenous promoter. (B) Complementation experiments of lrg-1(12-20) transformed with the indicated constructs. Transformants were grown at 37°C to determine the complementation capability of the respective construct. Bar, 200 μm.
MYC mis-localized to the hyphal cortex. Bar, 10 μm. (B) Localization data generated in the ApoTome modus indicate an apical vesicular–reticulate localization of GFP::LRG1 and a more diffuse localization of GFP::LRG1*. Bar, 5 μm. (C) GFP::LRG1 localized as a crescent at growing hyphal tips and at septae (left), whereas GFP::LRG1* containing the three mutated LIM domains did localize in a diffuse manner throughout the cell (right). Bar, 5 μm.

When we quantified the length of the GFP::LRG1 marked apical cortex relative to the growth rate of the tip, we observed a positive correlation (R² = 0.6204) between growth rate and size of the apical cap.

Septum formation in filamentous fungi requires the formation of actin rings as an initial step before cross wall polymerizing agent induced a morphological change of the hyphal tip after ~5 min. Nevertheless, the septal pore-associated localization of GFP::LRG1 remained unaffected by this treatment, indicating that this terminal association of LRG1 with the central region of the septum is independent of a functional actin cytoskeleton.

The Apical Localization of LRG1 Is Influenced by a Functional Microtubule System and Opposing Motor Proteins

We also tested the dependence of the localization of LRG1 on a functional microtubule cytoskeleton by growing GFP::LRG1 on 4 μg/ml nocodazole or 5 μg/ml benomyl. For both treatments, the apical LRG1 cap persisted as long as growth was observed, indicating that a functional microtubule cytoskeleton is dispensable for LRG1’s localization (data not shown). However, the size of the apical GFP::LRG1 cap was reduced by ca. 50% in inhibitor-treated cells when compared with untreated wild-type tips growing at comparable rates (Figure 5A), suggesting that a functional microtubule cytoskeleton is necessary for a stable accumulation of LRG1 at the hyphal tip.

The high rate of fungal tip extension requires efficient microtubule-based and motor protein-dependent transport systems for supplying the growing tip with all necessary growth supplies and regulatory factors (Steinberg, 2007). This transport toward the growing tip could be the reason for the observed microtubule-dependence of apical LRG1 localization. Thus we analyzed the impact of mutants defective in anterograde- (kinesin-1) and retrograde-directed (dynein) on apical GFP::LRG1 localization (Seiler et al., 1999; Zhang et al., 2003). We compared the growth rates of various ropy (= dynein/dynactin) strains in single as well as double mutants in combination with lrg-1(12-20) and found that ropy mutations partially suppressed lrg-1(12-20) (Figure 5B). In contrast to this common phenotype of lrg-1(12-20) and the Ndr pathway mutants cot-1(1) and pod-6(31–21), gul-1 that had been implicated in phosphatase-associated, COT1/POD6-antagonizing functions (Terenzi and Reissig, 1967; Seiler et al., 2006), only suppressed the growth defects of cot-1(1) and pod-6(31–21), but not those of lrg-1(12-20). Next, we tested for a genetic connection between lrg-1(12-20) and nkin, which had been shown to counteract dynein’s retrograde transport activity (Seiler et al., 1999; Zhang et al., 2003; Seiler et al., 2006). The growth rate of a lrg-1(12-20);nkin double mutant at semirestrictive temperature was reduced by 42% when compared with lrg-1(12-20) as the slower growing of the two parental strains, indicating a synthetic interaction between lrg-1(12-20) and nkin.
These data suggested that both opposing microtubule-dependent motor proteins are involved in localizing LRG1. Therefore, we determined the localization of GFP-tagged LRG1 in these motor mutants. As determined previously...
The majority of slow-growing hyphal tips of wild type displayed no GFP::LRG1 cap. The growth rates of the two motor mutants is ca. 20% of wild type (Seiler et al., 1997, 1999), and thus, a visible cap should be rarely visible, if the GFP::LRG1 localization is independent of the two motors. We observed apical caps in only 22% of wild-type tips at growth rates $<0.05$ µm/s (Figure 5C). This contrasted sharply with 81% of ro-1 tips growing at rates of $<0.05$ µm/s that displayed an apical GFP::LRG1 cap, while the number of GFP::LRG1 caps at growth rates $>0.05$ µm/s was reduced to 8% in the nkin background (Figure 5C). Thus the apical localization of LRG is influenced by opposing microtubule-dependent motor proteins. This localization dependence of LRG1 on both motor proteins and functional LIM domains was further supported when we introduced GFP::LRG1* in the ro-1 background. The resulting progeny closely resembled the $lrg-1$ deletion phenotype, with poorly growing and highly hyperbranched cells (Figure 5D). Interestingly, GFP::LRG1* localized in an aberrant cortical manner similar to what we have determined for the GAP domain alone (Figure 3A), suggesting that either of the two localization mechanism alone is sufficient for apical localization of LRG1, but disturbing of both targeting mechanisms results in abnormal localization of LRG1.

**LRG1 Is a RHO1-specific GAP Affecting Several Output Pathways of RHO1**

Given LRG1’s homology with Rho-GAP proteins, we determined, which of the six Rho-GTPases present in the *N. crassa* genome are regulated by LRG1. When we overexpressed the wild-type forms of all RHO proteins in $lrg-1(12-20)$, only RHO1 was capable of partially complementing the $lrg-1(12-20)$ growth defect. Also dominant active (Rho1 G15V) as well as dominant negative (Rho1 E41I) RHO1 alleles were able to overcome the $lrg-1(12-20)$ dependent growth defect initially and generated hyphal tips that are capable of apical extension (Figure 6A). Nevertheless, all growing transformants died within 2 d with swollen and lysed hyphae (data not shown). This death phenotype was also observed when we overexpressed the three RHO1 alleles in wild type and is thus likely the result of interfering with endogenous RHO1 function (Figure 6B). A deletion strain of rho-1 was available through the *Neurospora* Genome Project (Dunlap et al., 2007). Its heterokaryotic nature already suggested the essentiality of RHO1. When we plated asexually derived conidiospores on hygromycin-containing media, we detected a mixture of germinating and isotropically growing spores, indicating that RHO1 is essential for establishing polarity (Figure 6C). Similarly, we were unable to obtain viable hygromycin-resistant ascospores from wild type $\times \Delta$rho-1::hph + rho-1+ crosses (Figure 6C). The majority of the hygR ascospores did germinate apolarly, indicating that RHO1 is required for polarity establishment in both types spores. Rarely, we observed the formation of hygR colonies, which then showed a high rate of cell lysis, suggesting that suppressor mutation can overcome the rho-1 deletion, but cell wall integrity remains affected.

**Figure 5.** The localization of GFP::LRG1 is influenced by opposing microtubule-dependent motor proteins. (A) Growth rate and size of the GFP::LRG1 cap was determined in hyphae treated with 4 µM nocodazole for 2 min and compared with untreated control cells. Because the inhibitor treatment resulted in reduced growth rates, only slow-growing control cells with growth rates of up to 0.15 µm/s were included in the comparison. (B) $lrg-1(12-20)$, cot-1(1) and the respective double mutants with components defective in the dynein/dynactin subunits ro-1, ro-3, and ro-10 or in the phosphatase-associated factor gul-1 were cultivated for 3 d at 37°C. The increased colony diameter of $lrg-1(12-20);ro-1/-3/-10$ mutants compared with $lrg-1(12-20)$ indicate suppression of the GAP defect by loss of dynein/dynactin function. (C) Apical GFP::LRG1 caps were detected in 78% of wild-type tips with growth rates of $<0.05$ µm/s. This rate of GFP::LRG1 caps increased in mutants defective in the anterograde directed motor protein nkin to 91% and decreased to 19% in the mutants defective in the retrograde directed motor protein ro-1. (D) GFP::LRG1* in a ro-1 background localized along the cortex and the hyphal morphology of the double mutant closely resembled the deletion phenotype.
Next, we performed in vitro GTPase assays with the six bacterially expressed and affinity-purified His6::GST::Rho fusion proteins (Figure 6, D and E). All G-proteins had significant intrinsic GTP-GDP turnover rates, indicating that the purified enzymes were active. The addition of the GST::GAP domain of LRG1 stimulated only the GTPase activity of RHO1 (ca. 10-fold). None of the other GTPases exhibited any significant change in activity. Thus, both the genetic and the in vitro experiments identify LRG1 as a RHO1–specific GAP.

RHO1 is a bona fide regulatory subunit of the cell wall enzyme β1,3-glucan synthase in yeasts and filamentous fungi. Furthermore, it is activating the cell wall integrity MAP kinase pathway (Beauvais et al., 2001; Levin, 2005). Therefore, we tested the impact of defective LRG1 function on these signaling routes. We have recently described conditional mutants in gs-1, a regulator of β1,3-glucan synthase (Seiler and Plamann, 2003), and when we generated a lrg-1(12-20);gs-1(8-6) double mutant, it did display strong synthetic defects (Figure 7A). Its growth rate at permissive or semirestrictive temperature was reduced by 25 and 41%, respectively, when compared with lrg-1(12-20) as the slower growing of the two parental strains. At restrictive conditions, gs-1(8-6) generated growth-inhibited hyperbranched hyphae that were still capable of slow apical growth. In contrast, lrg-1(12-20);gs-1(8-6) formed apolarly growing spheres when germinated at 37°C. When germinated hyphae were transferred to restrictive conditions, chains of spherical-growing cells were generated within 8 h. Furthermore, these strains were less sensitive against caspofungin at semipermissive conditions (Figure 7B), a specific inhibitor of fungal β1,3-glucan synthesis (Denning, 2003), indicating hyperactive β1,3-glucan synthase in lrg-1(12-20) and gs-1(8-6). Although the growth rate of lrg-1(12-20) increased in the presence of caspofungin, hyphal morphology, and branching frequency was not different in treated versus untreated cells (Supplementary Figure S1).
The abnormal chitin distribution observed in \(lrg-1(12-20)\) (Figure 1A) could be a result of increased activity of the \(N.\ crassa\) PKC/MAK1 cell integrity pathway and subsequently the altered expression of additional cell wall enzymes. Therefore, we tested for altered activity levels of PKC in the GAP mutant. We observed a partial suppression of the \(lrg-1(12-20)\) growth defect on media supplemented with 1 \(\mu\)M staurosporine, a protein kinase inhibitor with highest specificity toward PKC (Figure 7C). Nevertheless, staurosporine is also inhibiting PKA (although with a ca. 10-fold lower affinity). Therefore, we tested the impact of 50 \(\mu\)M KT5720, a PKA-specific inhibitor, on the growth behavior of \(lrg-1(12-20)\), but observed no morphological changes (Figure 7C), indicating that PKC is hyperactive in \(lrg-1(12-20)\). This was further supported by growth tests on media supplemented with 60 nM cercosporamide, a highly specific inhibitor for PKC (Sussman \textit{et al}., 2004).

Next, we tested the activity of the cell integrity MAP kinase MAK1 by the use of phospho-specific antibodies against activated ERK-type MAPKs. No obvious differences were found in MAK1 phosphorylation pattern in wild-type extracts from cultures grown at 25 and 37°C. Stressing these cells by a temperature shift from 25 to 37°C for 20 min resulted in activation of cell integrity signaling and increased MAK1 activity (Figure 7D). In contrast, the MAK1 phosphorylation pattern in \(lrg-1(12-20)\) shifted to restrictive temperature for 20 min did not result in heat stress-induced phosphorylation of MAK1, suggesting that MAK1 regulation may be affected in \(lrg-1\) mutants. However, we did not detect increased MAK1 activity levels in \(lrg-1(12-20)\) after prolonged temperature shift (Figure 7D) or in \(\Delta lrg-1\) germinated on benomyl and panthotenic acid (data not shown) as one would predict for increased RHO1–MAK1 signaling. To test, whether the capacity of the MAK1 pathway to respond...
to stress signals is affected in \(lrg-1(12-20)\) in a general manner, wild type and \(lrg-1(12-20)\) were grown at 37°C and stressed by the addition of 7 mM H\(_2\)O\(_2\) for 20 min (Figure 7E). Both strains displayed identical activation patterns as detected by MAK1 phosphorylation, indicating that the response capacity of the MAK1 pathway is not affected in \(lrg-1(12-20)\). Taken together, these data indicate that RHO1–PKC signaling is increased in \(lrg-1(12-20)\), but also suggest that the downstream MAK1 MAP kinase pathway is only affected to a minor extent.

Furthermore, we determined that \(lrg-1(12-20)\) was hypersensitive against the actin depolymerizing drug latrunculin A, suggesting an altered actin cytoskeleton in the GAP mutant (Figure 7F). In summary, we suggest that LRG1 acts as a general GAP for RHO1 and regulates several RHO1-dependent signaling pathways in \(N.\ crassa\).

**Genetic Interactions of lrg-1 with the Ndr Kinase Mutant cot-1 Indicate Two Parallel Pathways for Polar Tip Growth**

The morphological defects of \(lrg-1(12-20)\) and of \(\Delta lrg-1\) were strikingly similar to those of the Ndr kinase mutant \(cot-1\) and its upstream regulating kinase \(pod-6\) (Yarden et al., 1992; Seiler et al., 2006). The common defects of these mutants are a block in apical tip extension and the generation of needle-like tips, the induction of multiple subapical branches, excessive and mislocalized chitin distribution, and a highly thickened cell wall. Furthermore, several recessive alleles of \(cot-1\), \(pod-6\), and \(lrg-1\) showed unlinked noncomplementation (Seiler and Plamann, 2003) and suggest LRG1 and COT1/POD6 either as components of two independent pathways that act in parallel to support apical tip growth or hint for a physical interaction of the three proteins. In contrast to a \(cot-1(1);pod-6(31–21)\) double mutant, which displayed defects identical to the two parental strains (Seiler et al., 2006), \(cot-1(1);lrg-1(12-20)\) or \(pod-6(31–21);lrg-1(12-20)\) double mutants were synthetically lethal (Figure 8A). Double mutant ascospores germinated at permissive conditions generated polar germ tubes, but grew in a compact and highly vacuolated manner with very slow tip extension rates and frequent lysis of hyphal tips or subapical regions. Germination at 37°C resulted in apolar germination and death of \(lrg-1(12-20);cot-1(1)\) ascospores. (bottom panel). Bars, 40 μm. (B) Double mutants of \(cot-1(1)\) and \(gs-1(8-6)\) did not display synthetic defects after shift to restrictive temperatures (left image) or when germinated under restrictive conditions (right image). Bar, 40 μm. (C) The growth behavior of \(cot-1(1)\) is identical to wild type on medium supplemented with 0.5 μM caspofungin. (D) \(cot-1(1)\) is not hypersensitive to the addition of 0.5 μM latrunculin A. (E) Total soluble protein of the indicated strains grown at 25 or shifted to 37°C for 10 h was extracted. The blot was probed with an anti-phospho-ERK antibody to detect increased MAK1 activity levels in \(cot-1(1)\) grown at restrictive conditions (top panel). To confirm equal loading the blot was reprobed with anti-tubulin antibody (bottom panel). One example of five consistent experiments is shown. (F) The PKC-specific inhibitors staurosporine and cercosporamide partially suppressed the tip extension defects of \(lrg-1(12-20)\), but not those of \(cot-1(1)\). The growth of wild-type cells was not affected by the mentioned conditions. One example of three consistent experiments is shown.

**Figure 8.** LRG1 acts in a pathway parallel to the Ndr kinase COT1 (A) \(lrg-1(12-20)\) and \(cot-1(1)\) displayed nearly identical defects with tip extension terminating with 2–10- and 10–30-μm-long pointed tips, respectively, when shifted from permissive to restrictive conditions for 12 h (top panel) or when germinated at 37°C for 15 h (middle panel). Double mutant ascospores germinated at 25°C produced compact, highly vacuolated and slow growing hyphae. Germination at 37°C resulted in apolar germination and death of \(lrg-1(12-20);cot-1(1)\) ascospores. (bottom panel). Bars, 40 μm. (B) Double mutants of \(cot-1(1)\) and \(gs-1(8-6)\) did not display synthetic defects after shift to restrictive temperatures (left image) or when germinated under restrictive conditions (right image). Bar, 40 μm. (C) The growth behavior of \(cot-1(1)\) is identical to wild type on medium supplemented with 0.5 μM caspofungin. (D) \(cot-1(1)\) is not hypersensitive to the addition of 0.5 μM latrunculin A. (E) Total soluble protein of the indicated strains grown at 25 or shifted to 37°C for 10 h was extracted. The blot was probed with an anti-phospho-ERK antibody to detect increased MAK1 activity levels in \(cot-1(1)\) grown at restrictive conditions (top panel). To confirm equal loading the blot was reprobed with anti-tubulin antibody (bottom panel). One example of five consistent experiments is shown. (F) The PKC-specific inhibitors staurosporine and cercosporamide partially suppressed the tip extension defects of \(lrg-1(12-20)\), but not those of \(cot-1(1)\). The growth of wild-type cells was not affected by the mentioned conditions. One example of three consistent experiments is shown.
that lrg-1 and cot-1/pod-6 share the same suppressors (Figure 5B), these genetic data indicate two parallel, COT1/POD6- and LRG1-dependent, pathways for apical tip extension.

Intrigued by these results, we tested for a potential connection between COT1 and RHO1 signaling and analyzed the RHO1 effector pathways that are affected by lrg-1. A highly thickened cell wall of cot-1(1) grown at 37°C described in electron microscopic sections (Corovits et al., 2000) and the excessive chitin distribution at sites of aberrant growth (Seiler et al., 2006) already indicated a defect in cell wall organization. However, we did not observe any synthetic interaction in a cot-1(1)/gs-1(8-6) double mutant (Figure 8B) and detected growth characteristics of cot-1(1) identical to wild type on media supplemented with caspofungin (Figure 8C), indicating no direct regulation of β1,3 glucan synthase by COT1. Furthermore, in contrast to the hypoactivity of lrg-1(12-20) to latrunculin A, we did not observe any difference of cot-1(1) compared with wild type when the strains were grown on media supplemented with 0.5 μM latrunculin A (Figure 8D). However, when we analyzed the activity of the cell wall integrity pathway in cot-1(1), we observed increased MAK1 activity levels in cot-1(1) cultures grown at restrictive temperature (Figure 8E), implying COT1 as a potential negative regulator of the MAK1 pathway. Interestingly, the cot-1(1) defect was not suppressed by the PKC inhibitors staurosporine and cercosporamide (Figure 8F), indicating that MAK1 MAP kinase activation is not induced via RHO1–PKC signaling. Taken together, these results establish distinct effects of LRG1 and of COT1 on the analyzed RHO1-dependent effector pathways.

**DISCUSSION**

Cellular polarity is a fundamental property of every cell, and fungal hyphae are among the most highly polarized cells known in nature. In a visual screen designed to identify factors critical for hyphal morphogenesis, we isolated lrg-1 as a component that is essential for apical tip extension and to restrict excessive branch formation in subapical regions of the hypha (Seiler and Plamann, 2003). The data presented here characterize LRG1 as a RHO1–specific GAP. In vitro, only the GTPase rate of RHO1 is stimulated significantly by LRG1, and the in vivo results indicate several misregulated RHO1 effector pathways in mutants lacking a functional GAP. Interestingly, but difficult to explain is that overexpression of wild type and of both dominant rho-1 versions is partially compensating the lrg-1 growth defects. Similar results were obtained for mammalian Cdc42 during cell proliferation, where the stable overexpression of the constitutively active G12V mutant did result in a dominant negative phenotype after prolonged growth (Vanni et al., 2005). Furthermore, the fact that lrg-1(12-20) cells, which are deficient for Rho-GAP activity, are defective in polarized growth, suggests that cycling of RHO1, rather than its GTP-bound form alone is important for its function. Thus, our results indicate that polar tip extension and the regulation of branch formation may involve cycling of N. crassa RHO1 to gain full activation, rather than its functioning as simple on/off switch.

Rho1 is a key regulator of hyphal growth and polarity and has been described as integral part of the β1,3-glucan synthase complex in yeasts and A. fumigatus (Arelanno et al., 1996; Beauvais et al., 2001; Levin, 2005). The observed caspofungin hyposensitivity and the genetic interaction of lrg-1(12-20) with gs-1(8-6) are consistent with increased β1,3-glucan synthase activity in lrg-1(12-20) and gs-1(8-6). Furthermore, the latrunculin A sensitivity suggest altered RHO1–actin signaling in lrg-1(12-20). Difficult to interpret are the data presented for the PKC/MAK1 pathway. The suppression of lrg-1(12-20) by the PKC specific inhibitors staurosporine and cercosporamide indicate hyperactivity of PKC in the GAP mutant. Nevertheless, downstream MAK1 activity is not enhanced, suggesting that the MAP kinase cascade is not regulated by LRG1. However, the MAK1 activation pattern in heat-stressed lrg-1(12-20) is different from wild type, supporting a regulatory function of the cell integrity pathway by LRG1. Thus, additional feedback mechanisms may regulate the activation of the PKC–MAK1 part of the cell integrity pathway in N. crassa.

Which of these RHO1 effector pathways may be responsible for the block in tip extension and the subapical hyperbranching observed in lrg-1? On the basis of data presented here, we do not believe that the altered activities of the PKC–MAK1 pathway are the primary defect. Nevertheless, the unregulated deposition of excessive chitin within the cell wall of lrg-1 may be a result transmitted through PKC. As demonstrated in budding yeast, cell wall stress can result in up to a 10-fold increase of the cell wall’s chitin content by stress-induced mobilization of Chs3p from chitosome vesicles to the plasma membrane. This stress signal is transmitted via Rho1p and Pkc1p, involving components of the cell integrity pathway, but does not include the downstream MAP kinase components (Valdivia and Schekman, 2003).

Loss of LRG1 results in increased glucan formation, which may interfere with the plasticity of the hyphal cell wall and could thus lead to the cessation of tip extension and the induction of branch formation in subapical regions of the hypha. This hypothesis is supported by a common hyperbranching phenotype of lrg-1(12-10) and gs-1(8-6) as a result of increased glucan deposition in the cell walls of both strains as monitored by the hyposensitivity to the glucan synthase inhibitor caspofungin. Interestingly is that the lrg-1(12-10)/gs-1(8-6) double mutant loose cellular polarity when hyphae are shifted to restrictive conditions and are unable to establish polarity when conidia are germinated at high temperature. This phenotype is reminiscent to conditional mutants of key regulators of the actin cytoskeleton such as bem-1 and cdc-24 (Seiler and Plamann, 2003) and suggests a tight connection between cell wall function and the actin cytoskeleton for apical tip extension and regulation of branch formation. Thus, we speculate that increased GS1 activity and altered actin organization are the major cause for the observed lrg-1 defects.

A clear homolog of LRG1 is also found in S. cerevisiae (E = 1e−11), but conflicting evidence about the Rho protein(s) and the Rho effector pathway(s) that are regulated by Lrg1p was reported. In vitro and yeast two hybrid analyses identified Lrg1p as a GAP for Rho1p, Rho2p, or Cdc42p or for combinations of these GTPases (Lorberg et al., 2001; Roumanie et al., 2001; Watanabe et al., 2001; Fitch et al., 2004). However, most genetic data link Lrg1p with Rho1p functions (Lorberg et al., 2001; Watanabe et al., 2001; Fitch et al., 2004; Varelas et al., 2006; Stewart et al., 2007), suggesting that Lrg1p is a Rho1p specific GAP in budding yeast. Furthermore, the effector pathways that are regulated by Lrg1p are discussed in a controversial manner. Two studies report enhanced MAP kinase activity (Lorberg et al., 2001; Stewart et al., 2007) in contrast to a report describing no effect on the MAP kinase (Watanabe et al., 2001). In addition, increased glucan synthase activity was reported for Δlrg-1 (Lorberg et al., 2001; Watanabe et al., 2001; Fitch et al., 2004), but no effect of Lrg1p on the Bni1p/actin branch of Rho1p signaling could be identified in any of these studies. In contrast,
Our double mutant analysis, nonalleleic noncomplementing N-terminus is resulting in the loss of function of LRG1 as dent of the functionality of the LIM domains. The abolish-distribution of LRG1 and thus allow growth that is indepen-port rates of NKIN and dynein result in a balanced directed transport. Thus we suggest that the opposing trans-branches that are induced by the temperature shift in mutants versus identical phenotypes. The major difference of the kinase dance is reduced in transport affected the localization of LRG1. The reduced reduced the apical LRG1 localization and that motor-driven with additional sites within the N-terminus of LRG1. We 2001; Brown and Turner, 2004). Another possibility could be similar to paxillin in focal adhesion complexes (Schaller, suggesting that LRG1 might act as a cytoskeletal organizer LRG1 homologues have much weaker similarities of E high sequence similarity of these domains with paxillin (E = 1e-28, whereas other LIM domain proteins aside from LRG1 homologues have much weaker similarities of E = 1e-14), suggesting that LRG1 might act as a cytoskeletal organizer similar to paxillin in focal adhesion complexes (Schaller, 2001; Brown and Turner, 2004). Another possibility could be the interaction of microtubule-dependent motor proteins with additional sites within the N-terminus of LRG1. We showed that treatment of cells with anti-microtubule drugs reduced the apical LRG1 localization and that motor-driven transport affected the localization of LRG1. The reduced retrograde transport of dynemin/dynactin mutations resulted in enriched apical localization of LRG1, whereas its abundance is reduced in nkin, which is defective for apical-directed transport. Thus we suggest that the opposing transport rates of NKIN and dynactin result in a balanced distribution of LRG1 and thus allow growth that is independent of the functionality of the LIM domains. The abolishment of both—the microtubule- and LIM domain-dependent—localization mechanisms by deleting the whole N-terminus is resulting in the loss of function of LRG1 as observed in LRG1761–1279 and by the introduction of LRG1* into the ro-1 background. Both conditions resulted cells re-ssembling the lrg-1 loss-of-function phenotype and in an aberrant cortical localization of the GAP. A similar dependence for correct localization on opposing motor proteins was also observed for cot-1 and pod-6 and suggest a general mechanism to supply all regions of fast growing, highly polar cells with the necessary components. In contrast to this microtubule-dependent distribution of LRG1 throughout the hypha, its localization as an apical cap during active growth is clearly F-actin–dependent, as determined in the latrunculin A inhibitor experiments.

In addition to this motor-dependent localization of LRG1 and COT1/POD6, mutants defective in the two kinases of the Ndr signaling network and in lrg-1 displayed nearly identical phenotypes. The major difference of the kinase mutants versus lrg-1 is the reduced length of the subapical branches that are induced by the temperature shift in lrg-1. Our double mutant analysis, nonalleleic noncomplementing alleles and common suppressors provide strong evidence that the COT1/POD6 complex is acting in parallel to LRG1/ RHO1 signaling. Genetic data in S. cerevisiae suggest that the COT1 homolog Cbk1p may negatively regulate the small GTPase Rho1p, although the mechanism of interaction re-mained open (Jorgensen et al., 2002; Schneper et al., 2004). A similar negative effect of COT1 on RHO1 signaling in N. crassa would explain the observed synthetic lethality of the lrg-1(l12-20);cot-1(1) double mutant. However, loss of COT1 function did not affect GS1 activity or the actin cytoskeleton, and the only RHO1 effector pathway that is altered in cot-1(1) is the MAK1 MAP kinase pathway (but not the upstream acting PKC). Thus, the molecular basis for the observed genetic interactions remains open. Interesting in this context is that a physical interaction between the Ndr kinase ORB6 and a different Rho-GAP has been proposed to exist in fission yeast based on two hybrid studies (Das et al., 2007). An indication that the connection between Rho GTPases and Ndr signaling is conserved between fungi and animals has been provided by studies in D. melanogaster and C. elegans (Zallen et al., 2000; Emoto et al., 2004). Thus, the genetic connection established, but also the distinctions between COT1/POD6 and LRG1/RHO1 signaling during hyphal growth, and the similarities of LRG1 to the focal adhesion organizer paxillin may pro-vide insights in the regulation of morphogenesis in other highly polar cells such as neurons.

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