Coordination of Cytokinesis and Cell Separation by Endosomal Targeting of a Cdc42-specific Guanine Nucleotide Exchange Factor in *Ustilago maydis*

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The small GTPase Cdc42 is a key regulator of cell polarity and cytoskeletal organization in most eukaryotic cells. In *Ustilago maydis*, Cdc42 and the guanine nucleotide exchange factor (GEF) Don1 regulate cytokinesis and cell separation. Don1 belongs to the FGD1 family of Cdc42-specific GEFs that are characterized by a C-terminal lipid-binding FYVE domain. Although the FGD1/frabin family of Rho-GEFs is evolutionary conserved from fungi to mammals the role of the FYVE domain for its biological function is unknown. Here, we show that the FYVE domain is specific for phosphatidylinositol-3-phosphate (PtdIns(3)P) and targets Don1 to endosomal vesicles. During cytokinesis asymmetric accumulation of Don1-containing vesicles occurs at the site of septation. We could show that FYVE-dependent localization is critical for the function of Don1 at normal expression levels but can be compensated for by overexpression of Don1 lacking a functional FYVE domain. Our results demonstrate that endosomal compartmentalization of a Cdc42-specific exchange factor is involved in the coordination of cytokinesis and cell separation.

INTRODUCTION

Small GTP-binding proteins of the Rho/Rac/Cdc42 family act as molecular switches in various biological processes, such as cytoskeleton reorganization, vesicular trafficking, and cell polarity (Jaffe and Hall, 2005). They cycle between two states: the active GTP-bound state and the inactive GDP-bound state. The switching between these states is regulated by interaction with accessory proteins. GTPase-activating proteins (GAPs) promote hydrolysis of GTP and thus trigger inactivation of the GTP-binding protein (Bernards and Settleman, 2004). Guanine nucleotide exchange factors (GEFs) catalyze the release of GDP by stabilizing the nucleotide-free transition state (Rossman *et al.*, 2005). This results in exchange of GDP by GTP because the latter nucleotide is much more abundant in the cytoplasm. Most GEFs specific for members of the Rho/Rac/Cdc42 family are characterized by the presence of the catalytic dbl homology (DH) domain and an associated lipid-binding pleckstrin homology (PH) domain (Rossman *et al.*, 2005). Although many different GEFs of the Dbl family are known, only little is known about their spatial and temporal regulation (Erickson and Cerione, 2004). Recent studies indicate that interaction of the PH domain with specific phosphoinositides seems to play a role not only for association to the plasma membrane but also for allosteric regulation of GEF activity (Baumeister *et al.*, 2006). Most members of the Dbl family contain additional domains that contribute to spatial and temporal regulation of GEF signaling. The mammalian Rac1-specific GEF

Tiam1 contains a second PH domain located in the N-terminal region (Habets *et al.*, 1994). Members of the FGD1/Frabin family are characterized by a C-terminal FYVE domain (Pasteris *et al.*, 1994; Obaishi *et al.*, 1998). FYVE domains consist of a cysteine-rich Zinc finger region, which mediates endomembrane association by interaction with phosphatidylinositol phosphates (Gaullier *et al.*, 1998; Patki *et al.*, 1998). Human FGD1 is involved in bone formation and its deficiency causes faciogenital dysplasia (Aarskog-Scott syndrome; Pasteris *et al.*, 1994; Gorski *et al.*, 2000). The *Caenorhabditis elegans* homolog Exc-5 plays a critical role in morphogenesis of excretory cells (Suzuki *et al.*, 2001). All members of the FGD1 family examined so far act as GEFs specific for Cdc42, the central regulator of cell polarity (Zheng *et al.*, 1996; Umikawa *et al.*, 1999; Etienne-Manneville, 2004; Hlubek *et al.*, 2008; Huber *et al.*, 2008). Although the role of the PH domain for GEF localization and activity has been studied in depth (Erickson and Cerione, 2004), the contribution of the lipid-binding FYVE domain for GEF signaling has not been addressed yet.

In the dimorphic fungus *Ustilago maydis* the FYVE domain containing GEF Don1 acts as key regulator of cell separation during budding growth (Weinzierl *et al.*, 2002; Hlubek *et al.*, 2008). *U. maydis* cells lacking Don1 are viable but fail to complete cytokinesis and form large cell clusters. Cell separation in *U. maydis* involves the consecutive formation of two distinct septa that delimit a fragmentation zone at which cell separation occurs by lysis of the connecting cell wall (Weinzierl *et al.*, 2002). The FYVE domain containing GEF Don1 is required for triggering the initiation of the secondary septum via specific activation of Cdc42 (Mahlert *et al.*, 2006; Hlubek *et al.*, 2008). Here, we show that the presence of the FYVE domain is critical for efficient targeting of the GEF Don1 to the site of septation. The FYVE domain mediates association of Don1 with endosomal vesicles, which accumulate asymmetrically at the daughter side of the primary septum. We propose that endosomal localiza-

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Abbreviations used: GEF, guanine nucleotide exchange factor.

tion of Don1 serves to coordinate secondary septum initiation with formation of the vacuolated fragmentation zone between mother and daughter cell.

MATERIALS AND METHODS

Plasmids, Strains, and Growth Conditions

Construction of plasmids was performed using standard procedures (Sambrook and Russell, 2001). *U. maydis* strain Bub8 (*a2 b4*) was used for all experiments (Schulz *et al.*, 1990). The *don1* strain has been described before (Weinzierl *et al.*, 2002). To generate truncated Don1 variants parts of the Don1 gene were replaced by homologous recombination. Deletion of *kin3* was performed by replacement of the complete open reading frame (ORF) with a hygromycin resistance marker. Correct integration of all replacement constructs was verified by Southern analysis.

Green fluorescent protein (GFP) fusions were constructed in plasmid p123, which carries an enhanced GFP (eGFP) reporter under control of the strong constitutive *otf* promoter (Spelling *et al.*, 1996). To generate mCherry fusion constructs, the eGFP ORF in p123 was replaced by the mCherry ORF (Shaner *et al.*, 2004). For colocalization analysis, a Yop1-GFP under control of its own promoter was introduced into strains expressing either Don1₃₃₁₋₁₀₁₄-mCherry or FYVE_{Don1}-mCherry. Detailed cloning procedures can be requested from the authors. Expression of all constructs was verified by Western blotting.

Protein Expression and Purification of the Don1 FYVE Domain

Protein expression of the Don1 FYVE domain was performed in strain Rosetta 2(DE3) (Novagen, Madison, WI) using plasmids derived from pGEX4T-1 (GE Healthcare, Waukesha, WI). Cells were grown overnight in autoinducing ZYM5052 medium at 22°C (Studier, 2005). Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 10 μ M ZnCl₂, 2.5 mM 2-mercaptoethanol), and lysed by two passages through a French press. After clearing the lysate by centrifugation, the supernatant was loaded on GSH Sepharose beads (GE Healthcare) and incubated at 4°C for 1 h. After removal of the supernatant, the beads were washed five times with lysis buffer and then eluted in elution buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 10% glycerol, 10 μ M ZnCl₂, 2.5 mM 2-mercaptoethanol, and 50 mM reduced glutathione). Elevation of the pH to 9.0 and the high level of glutathione were necessary to allow complete elution. Fractions containing protein were pooled and used immediately in protein-lipid overlay assays. As a positive control, the FYVE domain of murine Hrs1 was amplified from mouse cDNA and purified as described above.

Protein Lipid Overlay Assays

Protein lipid overlay assays (Dowler *et al.*, 2002) were performed using commercially available PIPStrips (Echelon Biosciences, Salt Lake City, UT) according to the manufacturer's instructions.

The PIPStrips were first blocked for 1 h in blocking solution (1% skimmed milk powder in TBS) at room temperature. Next, the PIPStrips were incubated

Figure 1. The lipid-binding FYVE domain is critical for the *in vivo* function of the Cdc42-specific GEF Don1. (A) Deletion analysis of the *don1* gene. The endogenous copy was replaced by constructs expressing C-terminally truncated GFP fusion proteins and transformants were scored for cell separation. +, more than 90% single cells; -, <10% single cells. Large parts of the C-terminus can be deleted without affecting cell separation. Removal of the FYVE domain results in loss of cell separation. Exchange of a single amino acid (R965A), which is important for lipid-binding activity of the FYVE domain interferes with Don1 function and results in loss of cell separation. (B) Western blot analysis of mutant strains demonstrates that all constructs are stably expressed. An mAb directed against GFP was used. (C) Wild-type strains are unicellular and grow by budding. Mutants deleted for Don1 ($\Delta don1$) exhibit a cytokinesis defect and form large clusters of nonseparated cells. The cell separation defect can be rescued by expression of full-length Don1-GFP (Don1). The last 263 amino acids of Don1 can be deleted without affecting its function (Don1 Δ C). Cells expressing Don1 constructs that either lack the lipid-binding FYVE domain (Don1 Δ FYVE) or carry a single amino acid exchange which abolishes lipid-binding (Don1^{R965A}) are unable to undergo cell separation. Scale bar, 10 μ m.

overnight at 4°C with purified GST-FYVE (2 µg/ml in blocking solution), washed three times with TBS-T, and incubated with HRP-conjugated anti-glutathione S-transferase (GST) antibody (GE Healthcare) at a dilution of 1:5000 in blocking solution for 1 h. After three washing steps, detection was performed using commercially available ECL reagents (GE Healthcare).

Staining Procedures

Staining of endocytic membranes using FM4-64 was performed as described (Vida and Emr, 1995). Cells were pulse-labeled at room temperature, washed with water, and then chased for 20 min at 30°C. At this time point, cells were fixed by incubation with 10 mM NaN₃ and directly analyzed by fluorescence microscopy. Cell walls were stained using Calcofluor White as described (Mahlert *et al.*, 2006).

Light Microscopy and Image Processing

For microscopic analysis, logarithmically growing cells were placed on agarose cushions and directly analyzed using a Zeiss Axiovert 200 microscope (Thornwood, NY). Images were taken using a cooled CCD camera (Hamamatsu Orca-ER, Bridgewater, NJ) with an exposure time of 100–1000 ms.

Image acquisition and deconvolution were performed using Improvision Velocity Software (Lexington, MA). Image analysis and processing was performed using NIH ImageJ (<http://rsb.info.nih.gov/ij/>; Abramoff *et al.*, 2004).

RESULTS

The FYVE Domain Is Critical for Don1 Function during Cytokinesis

Budding *U. maydis* cells defective for the Cdc42-specific GEF Don1 form large clusters of nonseparated cells (Figure 1C; Weinzierl *et al.*, 2002). We have used this clearly discernible and nonessential phenotype to study the role of the FYVE domain for the function of Don1 *in vivo*. To this end we replaced the endogenous copy of *don1* by a series of deletion mutants encoding C-terminally truncated versions (Figure 1A). All constructs were expressed under control of the endogenous *don1* promoter and were fused at their C-terminal ends to GFP to follow the intracellular localization of the truncated proteins. Cells expressing full-length Don1-GFP displayed normal cell separation indicating that C-terminal fusion to GFP did not interfere with biological function (Figure 1C). To test whether all constructs are stably expressed, we performed Western blot analysis using an mAb against GFP. All fusion proteins appeared to be stable and were present in amounts similar to the full-length protein (Figure 1B). GFP fluorescence, however, was below detection level for all constructs, indicating that the concentration of Don1 is very low (data not shown). This is in accordance to the expression data obtained for Don1 by microarray analysis (J. Kämper, personal communication).

Several independent transformants were scored for proper cell separation (Figure 1A and see Supplemental Table S1 for quantification). The region located C-terminally of the FYVE domain could be removed without affecting Don1 function (Figure 1C, Don1ΔC). However, if the FYVE domain was deleted, large clusters of nonseparated cells were observed (Figure 1C, Don1ΔFYVE). This indicates that the C-terminus including the conserved FYVE domain is critical for the function of Don1 during cytokinesis and cell separation.

FYVE domains are lipid-binding domains that recognize preferentially phosphatidylinositol-3-phosphate (PtdIns(3)P), a phospholipid characteristic for endosomal and vacuolar membranes (Gaullier *et al.*, 1998; Patki *et al.*, 1998; Gillooly *et al.*, 2000). To determine the lipid-binding specificity of the Don1 FYVE domain, we performed a protein–lipid overlay assay (Dowler *et al.*, 2002) using recombinant GST-FYVE_{Don1} fusion protein. The FYVE domain of murine Hrs1, which binds to PtdIns(3)P, served as a control (Sankaran *et al.*, 2001). This assay clearly demonstrated that the FYVE do-

Figure 2. The Don1 FYVE domain binds specifically to PtdIns(3)P. Protein-lipid overlay assay using GST fusion proteins demonstrates that the Don1 FYVE domain binds with high specificity to PtdIns(3)P. As positive control, the FYVE domain of murine Hrs1 was used.

main of Don1 binds with high specificity to PtdIns(3)P and does not recognize other phospholipids (Figure 2).

To test whether the lipid-binding property of the Don1 FYVE domain is critical for function, we introduced a single amino acid exchange into the lipid-binding pocket of the FYVE domain (R965A). The corresponding mutation has been described for the FYVE domains of Hrs1 and EEA1 (R183A and R1375A, respectively) and interferes strongly with lipid binding without affecting the structural integrity of the FYVE domain (Gaullier *et al.*, 2000; Raiborg *et al.*, 2001). Don1^{R965A} was unable to trigger cell separation (Figure 1, A and C), thus supporting our notion that the lipid-binding activity of the FYVE domain is critical for Don1 function.

Don1 Accumulates at the Site of Cell Separation

To determine the intracellular localization of Don1, we expressed full-length and truncated Don1-GFP fusion proteins (Figure 3A) under control of the constitutive *otef* promoter (Spellig *et al.*, 1996). Stable expression of all constructs was confirmed by Western blot analysis (data not shown). Localization of fusion proteins was analyzed by fluorescence microscopy and quantified by counting dividing cells. In Figure 3 representative images are shown with the typical localization pattern detected in at least 85% of dividing cells ($n \geq 40$). For full-length Don1-GFP fusion protein, we observed an accumulation at the site of septation, although the level of fluorescence was very weak (Figure 3B, Don1-GFP). Deletion of the C-terminal 327 amino acids, which encompass a PEST region, resulted in an increase of fluorescence intensity but did not alter specific localization during cytokinesis (Figure 3B, Don1_{1–1014}-GFP). PEST sequences are signals for protein degradation and have also been detected in Rho-GEFs (Rechsteiner, 1990; Mertens *et al.*, 2003). Expression of Don1_{1–807}-GFP, which also lacks the FYVE domain, resulted in cytoplasmic fluorescence without accumulation at the site of septation (Figure 3B, Don1_{1–807}-GFP). To improve visualization we also deleted the N-terminus of Don1, which contains two additional PEST sequences. Expression of constructs Don1_{332–807}-GFP and Don1_{332–1014}-GFP resulted in strong fluorescence without affecting the respective

Figure 3. The Don1 FYVE domain is necessary and sufficient for targeting of Don1 to the site of septation. (A) Different fragments of Don1 were fused to GFP and expressed under control of the constitutive *otef* promoter. Cell separation was scored by complementation of a *don1* mutant strain. For quantification, see Supplemental Table S1. +, more than 90% single cells; -, <10% single cells; and +/-, a value between 10 and 90% single cells. (B) Subcellular localization of Don1-GFP fusion constructs was analyzed by fluorescence microscopy. Accumulation at the site of septation is indicated by arrowheads. Scale bar, 10 μ m.

specific localization pattern (Figure 3B, Don1₃₃₂₋₁₀₁₄ and Don1₃₃₂₋₈₀₇). This significant increase in protein stability due to removal of the N-terminal PEST domains was also confirmed by Western blot analysis (data not shown). Although the FYVE-domain containing Don1₃₃₂₋₁₀₁₄ protein accumulated at the site of septation, Don1₃₃₂₋₈₀₇, which lacks the FYVE domain, showed a purely cytoplasmic distribution. These data indicate that the presence of the FYVE domain is required to target Don1 to the septation site.

Next we asked whether the FYVE domain is also sufficient to direct Don1 to the site of septation. To this end, we

expressed the isolated FYVE domain as GFP fusion protein. This construct showed a very prominent accumulation at the septation site (Figure 3B, FYVE_{Don1}-GFP), indicating that the FYVE domain is both necessary and sufficient to target Don1 to the site of cell separation.

We noted that all constructs that accumulated at the site of septation during cytokinesis could also be found on endomembranes, in particular vacuolar membranes and rapidly moving vesicles. To test whether the lipid binding activity of the FYVE domain is critical for the specific localization pattern of Don1, we expressed the full-length Don1^{R965A}-GFP and the isolated FYVE^{R965A}-GFP domain, both carrying the single amino acid exchange in the FYVE domain. Neither of these constructs did accumulate at the site of septation, and both displayed a purely cytoplasmic distribution (Figure 3B, Don1^{R965A} and FYVE^{R965A}). In addition, both constructs did not show any association with endomembranes and could not be found at moving vesicles.

All overexpression constructs were also tested for complementation of the cell separation defect of $\Delta don1$ mutants (Figure 3A and Supplemental Table S1 for quantification). Interestingly, overexpression of Don1₁₋₈₀₇-GFP, which lacks the lipid-binding FYVE domain resulted in a partial rescue of the cell separation defect (Figure 2A and Supplemental Table S1). This was quite unexpected because expression of the respective deletion construct under control of its endogenous promoter was not able to trigger cell separation (Figure 1A). Overexpression of N-terminally truncated Don1₃₃₂₋₈₈₇-GFP, which lacks both the FYVE domain and the N-terminal PEST sequences, resulted even in full complementation (Figure 3A and Supplemental Table S1). The same complementation upon overexpression was observed for Don1^{R965A}, which carries the point mutation in the FYVE domain. Thus overexpression of the catalytic DH and its associated PH domain was sufficient to trigger cell separation. Together, these data suggest that the FYVE domain is involved in specific targeting of Don1 but is apparently not essential for Don1 GEF activity.

The Lipid-binding FYVE Domain Localizes Don1 to Endosomal Vesicles

We could show that the Don1 FYVE domain binds specifically to PtdIns(3)P. This phospholipid is characteristic for endosomal and vacuolar membranes (Gaullier *et al.*, 1998; Patki *et al.*, 1998; Gillooly *et al.*, 2000), suggesting that the FYVE domain recruits Don1 to the endosomal compartment. To test this hypothesis, we performed colocalization experiments. To this end we stained cells with the steryl dye FM4-64, which inserts into the outer layer of the plasma membrane and is internalized via the endocytic pathway (Vida and Emr, 1995). Both, Don1₃₃₂₋₁₀₁₄-GFP and FYVE_{Don1}-GFP clearly colocalized with FM4-64 at both endosomal and vacuolar membranes (Figure 4A). Next, we coexpressed the endosomal t-SNARE Yup1, the *U. maydis* Vam7p homolog (Wedlich-Söldner *et al.*, 2000). Colocalization of Yup1-GFP was observed both with mCherry-tagged Don1₃₃₂₋₁₀₁₄ and FYVE_{Don1} (Figure 4B). Together these data indicate that the lipid-binding FYVE domain recruits the GEF Don1 to the endosomal and vacuolar compartment.

In *U. maydis*, endosomal vesicles are transported along microtubules by the Kif1A/Unc104-like kinesin motor protein Kin3 (Wedlich-Söldner *et al.*, 2002). We observed fast bidirectional movement of vesicles carrying either Don1₃₃₂₋₁₀₁₄-GFP or FYVE_{Don1}-GFP in wild-type cells (Figure 5 and Supplemental Movies S1 and S2). Next, we tested whether the motility of these Don1-carrying vesicles is affected in $\Delta kin3$ mutants. Long distance movement of these

Figure 4. The Don1 FYVE domain confers endosomal localization. (A) Don1₃₃₂₋₁₀₁₄-GFP and FYVE_{Don1}-GFP are associated with endocytic membranes stained with the sterol dye FM4-64. (B) Don1₃₃₂₋₁₀₁₄-mCherry and FYVE_{Don1}-mCherry localize to endosomal vesicles labeled by the endosomal t-SNARE Yup1-GFP. Images in B were subjected to iterative deconvolution.

vesicles was largely abolished in strains lacking the motor protein Kin3 (Figure 5 and Supplemental Movies S3 and S4). Although the fusion proteins were mostly found in nonmotile vesicle aggregates, we observed some residual motility, most likely due to dynein driven transport as has been described (Wedlich-Söldner *et al.*, 2002). Together these data support our notion that Don1 associates with endosomal vesicles. Interestingly, *kin3* mutants are not affected in formation of the secondary septum (data not shown), indicating that the kinesin-driven long distance transport of Don1-carrying endosomal vesicles is not critical for Don1 function.

Because PtdIns(3)P-binding activity of the FYVE domain is required both for function and localization of Don1, we asked whether depletion of intracellular PtdIns(3)P levels by inhibition of PI3 kinase activity would result in a cell separation defect. Interestingly, treatment of *U. maydis* cells with high doses of the PI3 kinase inhibitor wortmannin neither affected cell separation nor association of the FYVE domain

with endosomal vesicles (see Supplemental Figure S1). A similar observation was made with LY294002, another known PI3K inhibitor (data not shown). It should be noted that sensitivity of PI3 kinases for wortmannin and LY294002 has been demonstrated only for metazoan cells, whereas fungal PI3 kinases, such as Vps34 in *Saccharomyces cerevisiae* and its homologue in *Schizosaccharomyces pombe*, were found to be resistant to both wortmannin and LY294002 (Stack and Emr, 1994; Takegawa *et al.*, 1995). Therefore, we consider it likely that also the *U. maydis* PI3 kinase is insensitive to these inhibitors.

Dynamic Localization of Don1-carrying Endosomal Vesicles during Cytokinesis

We next examined the temporal and spatial distribution of Don1-carrying endosomal vesicles during cytokinesis and cell separation. Before formation of the primary septum Don1₃₃₂₋₁₀₁₄-GFP-carrying vesicles could pass the mother-bud neck in both directions (not shown). During closure of the primary septum, vesicles accumulated at both sides of the cross-wall (Figure 6, top panel). Although the vesicles at the mother side disappeared, the accumulation of vesicles at the daughter side remained stable, resulting in a collar-shaped structure (Figure 6, middle panel). On formation of the secondary septum these Don1-carrying vesicles were trapped to a large extent in the fragmentation zone between primary and secondary septum (Figure 6, bottom panel). The same spatial and temporal distribution of vesicles was observed using the endosomal t-SNARE Yup1 as a marker. This corroborates that cell separation in *U. maydis* involves a subtle intracellular choreography of endosomal trafficking and vesicle accumulation.

DISCUSSION

In this communication we show that the Cdc42-specific GEF Don1 is recruited to endosomal vesicles that accumulate at the site of septation during cytokinesis. Membrane association of Don1 is mediated by the lipid-binding FYVE domain, which recognizes specifically PtdIns(3)P. Under endogenous expression levels endosomal localization of Don1 is critical for its function. Thus, we propose that compartmentalization of the Cdc42-GEF Don1 contributes to its function and serves to coordinate septum initiation and cell separation.

The Cdc42-specific Exchange Factor Don1 Localizes to Endosomes

We have examined the intracellular dynamics of the Cdc42-specific GEF Don1 during cytokinesis in the dimorphic fun-

Figure 5. Fast bidirectional movement of Don1-containing vesicles depends on the Kin3 motor protein. Wild-type and *kin3* mutant cells were transformed with constructs expressing Don1₃₃₂₋₁₀₁₄-GFP and FYVE_{Don1}-GFP and analyzed using time-lapse fluorescence microscopy. Significant movement of Don1₃₃₂₋₁₀₁₄-GFP and FYVE_{Don1}-GFP was observed only in wild-type cells but not in $\Delta kin3$ mutants. The movement of single vesicles was tracked and is indicated by arrows. Shown are frames of Supplemental Movies S1 to S4.

Figure 6. Daughter-cell-specific accumulation of endosomal vesicles carrying Don1 during cytokinesis. Cells expressing either Don1₃₃₂₋₁₀₁₄-GFP or Yop1-GFP were analyzed by fluorescence microscopy. Cell wall deposition during septum formation was followed by staining with calcofluor white (shown in the false color red). During formation of the primary septum endosomes accumulate at both sides of the septum (top panel). After closure of the primary septum, endosomes accumulate solely at the daughter side of the septum (middle panel). During formation of the secondary septum, endosomal vesicles are enclosed within the developing fragmentation zone between the two septa. Intensity plots were calculated from the micrographs along the indicated line using ImageJ. For quantification, dividing cells with a visible primary septum were scored, and the fraction of cells exhibiting the respective vesicle distribution is indicated.

gus *U. maydis*. Don1 is a fungal member of the FGD family of Rho-GEFs, which is characterized by the presence of a lipid-binding FYVE domain. The FYVE domain of Don1 binds with high specificity to PtdIns(3)P, just as the majority of FYVE domains characterized so far (Misra *et al.*, 2001; Sankaran *et al.*, 2001; Cho and Stahelin, 2004). Here we show that the FYVE domain is necessary and sufficient to target Don1 to fast-moving endosomal vesicles. During cytokinesis, these vesicles accumulate at the division site.

Just recently, a similar endosomal localization has been observed for FGD2, another member of FGD1/frabin family. FGD2 is expressed in antigen-presenting cells and localizes to early endosomes and membrane ruffles (Huber *et al.*, 2008). The biological function of this membrane association is still unknown, and expression of FGD2 lacking a functional FYVE domain still results in activation of JNK1 (Huber *et al.*, 2008). Interestingly, FGD1 and frabin are both associated with the subcortical actin cytoskeleton (Estrada *et al.*, 2001; Kim *et al.*, 2002), and neither FGD1 nor frabin depends on the FYVE domain for intracellular localization. Nevertheless, the FYVE domain appears to be critical for function for frabin, because under low expression levels frabin constructs lacking this domain are unable to trigger the formation of filopodia (Ono *et al.*, 2000).

Although under endogenous expression levels of Don1 the lipid-binding FYVE domain is critical for function, overexpression of Don1 can bypass this requirement. This suggests that membrane association of Don1 is not absolutely required for its function during cytokinesis but presumably serves to increase the local concentration of Don1 at the site of its action. Overexpression of delocalized Don1 is expected to result in global activation of Cdc42, which is consistent with the fact that also expression of constitutive active Cdc42^{Q61L} is sufficient to trigger septum initiation in the absence of Don1 (Mahlert *et al.*, 2006). These data suggest that yet unknown landmark proteins determine the site of septation and that localization of Don1 is not absolutely required for this process. However, at endogenous expres-

sion levels targeting of Don1 appears to be prerequisite for localized activation of Cdc42 during cytokinesis.

Accumulation of Endosomal Vesicles during Cytokinesis and Cell Separation

Here, we could show that cytokinesis and cell separation in *U. maydis* is accompanied by a highly asymmetric distribution of endosomal vesicles. Interestingly, recent studies in both mammalian and plant systems have implicated a role for endosomal vesicles in cytokinesis and cell abscission (Baluska *et al.*, 2006; Montagnac *et al.*, 2008). In plants, PtdIns(3)P-rich vesicles accumulate at the growing ends of the newly formed cell plate, suggesting that they play a role in cell wall synthesis during cytokinesis (Vermeer *et al.*, 2006). Interestingly, in our fungal system the observed strong accumulation of PtdIns(3)P-rich vesicles during cytokinesis appears to play no role in cell wall synthesis but may be involved in the breakdown of the cell wall during cell abscission because they give rise to the future fragmentation zone (O'Donnell and McLaughlin, 1984). In animal cells, the ESCRT complex, which constitutes the endosomal sorting machinery, is recruited during cytokinesis to the midbody by interaction with the centrosomal protein Cep55 (Carlton and Martin-Serrano, 2007). Although it was reported microtubule-transported vesicles accumulate in an asymmetric manner at one side of the midbody (Gromley *et al.*, 2005), recent data show that both daughters contribute to vesicle accumulation at the cleavage furrow (Goss and Toomre, 2008).

Compartmentalization of the RhoGEF Don1 Coordinates Septum Formation with Cell Separation

The function of the Don1/Cdc42 signaling module lies in the initiation of secondary septum formation during cytokinesis (Weinzierl *et al.*, 2002; Mahlert *et al.*, 2006). This raises the problem of how the endosome-localized Don1 can trigger Cdc42 signaling. Cdc42 is prenylated at its C-terminal cysteine residue and thereby predominantly localizes to the

plasma membrane (Ziman *et al.*, 1993). Thus, efficient activation of Cdc42 would require that the endosomally localized Don1 interacts directly with plasma-membrane-localized Cdc42 to catalyze GDP/GTP exchange. This interaction probably takes place within the mother-bud neck where endosomes are tightly stacked and are thereby forced into close proximity to the plasma membrane. Alternatively, it is also feasible that activation of Cdc42 occurs on endosomal vesicles. In both cases a zone of active Cdc42 is formed in the mother-bud neck, which then may trigger the formation of a contractile actomyosin ring that precedes secondary septum formation (Böhmer *et al.*, 2008). Preliminary data indicate that overexpression of Don1 is able to recruit Cdc42 to endosomal vesicles (data not shown). This mechanism would deliver both Don1 and Cdc42 concomitantly to the septation zone. Interestingly, it was recently described that activation of Rac1 can occur on early endosomes where the exchange factor Tiam1 is also recruited (Palamidessi *et al.*, 2008).

Endomembrane targeting of Rho-GEFs is likely to be important also in other systems. Notably, the Don1/FGD1 related GEF Exc-5 is essential for the development of the single excretory cell in *C. elegans* (Suzuki *et al.*, 2001). This largest cell of *C. elegans* contains a complicated intracellular network of tubular canals that connects to the excretory duct. Exc-5 is found along the tubular system, where it coordinates the growth of the apical and basal sides of these canals (for review see Buechner, 2002). Thus it is tempting to speculate that the tubular network is derived from endosomal origin. Endomembrane-specific activation of Cdc42 has been observed in human cells for the Dbl family GEFs Dbl and Ost, which both contain a Sec14-like lipid-binding domain (Ueda *et al.*, 2004).

In all these processes it might be the specific compartmentalization of Cdc42-specific GEFs that guarantees the temporal and spatial coordination of vesicle targeting and fusion processes. Our findings in the fungal model system *U. maydis* thus provide new insights into the intracellular dynamics of Cdc42 signaling.

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