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Localization and role of MYO-1, an endocytic protein in hyphae of *Neurospora crassa*

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ABSTRACT

The subapical endocytic collar is a prominent feature of hyphae of *Neurospora crassa*. It comprises a dynamic collection of actin patches associated with a number of proteins required for endocytosis, namely, ARP-2/3 complex, fimbrin, coronin, etc. We presently show that MYO-1 is another key component of this endocytic collar. A *myo-1* sequence was identified in the genome of *N. crassa* and used it to generate a strain with a *myo-1-sgfp* allele under the *ccg1* promoter. Examination of living hyphae by confocal microscopy, revealed MYO-1-GFP located mainly as a dynamic collection of small patches arranged in collar-like fashion in the hyphal subapex. Dual tagging showed MYO-1-GFP partially colocalized with two other endocytic proteins, fimbrin and coronin. MYO-1 was also present during septum formation. By recovering a viable strain, albeit severely inhibited, after deletion of *myo-1*, it was possible to investigate the phenotypic consequences of the elimination of MYO-1. Deletion of *myo-1* caused a severe reduction in growth rate (95%), near absence of aerial mycelium and no conidiation. A reduced uptake of the lipophilic dye FM4-64 indicated a deficiency in endocytosis in the $\Delta myo-1$ mutant. Hyphae were produced by the $\Delta myo-1$ mutant but their morphogenesis was severely affected; hyphal morphology was distorted displaying irregular periods of isotropic and polarized growth. The morphological alterations were accompanied, and presumably caused, by a disruption in the organization and dynamics of a myosin-deprived actin cytoskeleton that, ultimately, compromised the stability and function of the Spitzenkörper as a vesicle supply center.

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1. Introduction

The internalization of plasma membrane by endocytosis is an important process in eukaryotic cells. It can serve for recycling membrane and proteins and for down-regulation of cell-surface receptors (Kaksonen et al., 2003). In *Saccharomyces cerevisiae*, actin is essential for plasma membrane invagination (Munn, 2001; Smith et al., 2001); treatment with latrunculin A (Ayscough, 2000) or interfering with actin dynamics (Munn, 2001) blocks endocytosis. In filamentous fungi, several studies have revealed the importance of actin in apical growth and hyphal shape maintenance (Echauri-Espinosa et al., 2012; Harris and Momany, 2004; Torralba et al., 1998; Virag and Griffiths, 2004). The actin present in the Spitzenkörper (Spk) is believed to participate in the delivery of vesicles (Bartnicki-García, 2002; Harris and Momany, 2004; Hasek and Bartnicki-García, 1994). The Spk is supposed to act as a vesicle supply center (VSC) coordinating the delivery of secretory

vesicles to the growing tip (Bartnicki-García et al., 1989). While actin patches located in the subapical region have profuse endocytic uptake (Araujo-Bazan et al., 2008; Berepiki et al., 2010; Delgado-Alvarez et al., 2010; Taheri-Talesh et al., 2008; Upadhyay and Shaw, 2008).

Hyphal extension involves continuous discharge of secretory vesicles at the apex to maintain the high rates of polar growth characteristic of filamentous fungi (Bartnicki-García, 2002). It has been calculated that the continuous deposition of plasma membrane at the hyphal tip generates an excess of membrane (Bartnicki-García, 2015). Thus making endocytosis a necessary mechanism to recycle membrane and its content (Atkinson et al., 2002; Fischer-Parton et al., 2000; Penalva, 2005, 2010). There is increasing evidence of the localization of endocytic components in the hyphal subapex and the importance of the endocytic collar in fungal growth and morphogenesis in hyphal fungi (Araujo-Bazan et al., 2008; Berepiki et al., 2010; Delgado-Alvarez et al., 2010; Taheri-Talesh et al., 2008; Upadhyay and Shaw, 2008). The accumulation of such evidence led to propose an apical recycling model (Penalva, 2010; Shaw et al., 2011; Steinberg, 2014). Membrane recycling takes place in a subapical zone by an endocytic

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collar composed of actin patches and several actin-binding proteins (ABPs). In *N. crassa* fimbrin, ARP-2/3 complex (Delgado-Alvarez et al., 2010) and coronin (Echauri-Espinosa et al., 2012) have been reported as part of the endocytic collar.

A large number of the proteins involved in yeast endocytosis have been well characterized (Idrissi and Geli, 2014; Kaksonen et al., 2003). The last step of the endocytic patch formation is the actin assembly promoted by the Arp2/3 complex (Kaksonen et al., 2003; Sirotkin et al., 2010), which activity is regulated by different actin nucleation promoting factors (NPF). In budding and fission yeast, four classes of NPF have been described, each has a distinct role in Arp2/3 regulation, WASP (Las17p in budding yeast and Wsp1 in fission yeast), myosin class I (Myo3/5 in budding yeast and Myo1 in fission yeast), Esp15-like Pan1, and Abp1 (Idrissi et al., 2008; Kaksonen et al., 2003).

Class I myosins is a family of proteins widely found in eucaryotes (Kim and Flavell, 2008; Sellers, 2000). The budding yeast *S. cerevisiae* possesses two homologous, functionally redundant myosins class I genes MYO3 (Goodson and Spudich, 1995) and MYO5 (Geli and Riezman, 1996). Deletion of either gene results in defects in endocytosis (Geli and Riezman, 1996; Goodson et al., 1996) and in the redistribution of actin patches to polarization sites during growth (Smith et al., 2001). Myo5p interacts with the NPFs Las17p (Idrissi et al., 2012, 2008; Naqvi et al., 1998) and Bbc1p (Lechler et al., 2000; Pruyne and Bretscher, 2000) to regulate actin nucleation via the Arp2/3 complex. Myo5p was shown to be present in patches, at the start of the invagination the endocytic vesicle (Anderson et al., 1998; Smith et al., 2001). The motor and actin nucleating activity of Myo5p are required for the internalization of the endocytic vesicle (Idrissi et al., 2008; Sun et al., 2006). As in budding yeast, the Myo1 of fission yeast activates the Arp2/3 complex (Kovar et al., 2011).

In *Candida albicans* CaMyo5p, its only class I myosin, is required for the actin patch organization at the tip and for hyphal formation (Oberholzer et al., 2002). In *Aspergillus* myosin class I is an essential gene, *myoA* in *A. nidulans* (McGoldrick et al., 1995) and *aipB* in *A. oryzae* (Matsuo et al., 2013). A conditional mutant of *myoA* in *A. nidulans*, formed enlarged cells, incapable of hyphal extension,

and displayed a reduced level of polarized growth and secretion under repressive conditions (Yamashita and May, 1998).

Although the localization of endocytic components in the hypha sub-apex was clearly demonstrated (Delgado-Alvarez et al., 2010; Penalva, 2010; Upadhyay and Shaw, 2008), the physiological importance of endocytosis in hyphal growth is not fully understood. In this study, we identified a single class I myosin (*myo-1* gene) in the genome of *N. crassa* and used it to generate a strain with a *myo-1*-GFP allele and a Δ *myo-1* mutant. We demonstrated that deletion of *myo-1* gene is not lethal although the mutant exhibits aberrant phenotype, including severe reductions in growth rate, near absence of aerial mycelia and total absence of conidia. Our findings showed the importance of MYO-1 in the stability of the actin cytoskeleton and in maintaining normal hyphal morphogenesis.

2. Materials and methods

2.1. Strains and growth conditions

Strains used in this work are listed in Table 1. All strains were maintained in Vogel's minimal medium (VMM) with 1.5% sucrose. When needed, L-histidine (200 µg/ml) or hygromycin B (200 µg/ml) was added. All strain manipulations were performed according to standard techniques (Davis, 2000).

2.2. *myo-1* deletion and cloning

Genomic DNA from *N. crassa* 4200 strain was used as template to produce a *myo1* gene replacement construct by double joint PCR (Yu et al., 2004). The 5' *myo1* fragment was obtained with primers 5For-*myo1* and 5Rev-*myo1* (see Table 1). The 3' *myo1* fragment was amplified with primers 3For-*myo1* and 3Rev-*myo1*. The hygromycin *hph* selective marker was amplified with primers *hph*-F and *hph*-R, using plasmid PCN44 as template. The three fragments were purified, mixed and used in a fusion PCR with primers NestFor-*myo1* and NestRev-*myo1*. The final 3927 bp *myo1*-*hph*-*myo1* cassette was purified and used to transform *N. crassa* strain

Table 1
Neurospora crassa strains, and oligonucleotides and plasmids used in this study.

Name	Genotype	Reference
4200	ORS-SL6a	FGSC#4200
FGSC-9717	Δ <i>mus51::bar+</i> ; <i>mat A his-3-</i>	FGSC#9717
FGSC-9718	Δ <i>mus51::bar+</i> ; <i>mat a</i>	FGSC#9718
TRM08-DD02	<i>mat a his-3+::Pccg-1-fim-1-sgfp+</i>	Delgado-Alvarez et al. (2010)
TRM25-OC18	<i>mat A his-3+::Pccg-1-crnl-1-mCherry+</i>	Echauri-Espinosa et al. (2012)
TRM49-OC30	<i>mat a his-3+::Pccg-1-lifeact-egfp+</i>	Delgado-Alvarez et al. (2010)
TRM-DD06	<i>mat A his-3+::Pccg-1-fim-1-mCherry+</i>	This work
TRM-FL01	Δ <i>myo-1::hph+</i> ; Δ <i>mus51::bar+</i> ; <i>mat a</i>	This work
TRM-FL02	Δ <i>mus51::bar+</i> ; <i>mat A his-3+::Pccg-1-myo-1-sgfp+</i>	This work
TRM-FL03	Δ <i>myo-1::hph+</i> ; <i>mat a his-3+::Pccg-1-lifeact-egfp+</i>	This work
TRM-FL04	Δ <i>myo-1::hph+</i> ; <i>mat a his-3+::Pccg-1-fim-1-sgfp+</i>	This work
TRM-FL05	<i>mat A his-3+::Pccg-1-crnl-1-mCherry+</i> ; <i>his-3+::Pccg-1-lifeact-egfp+</i>	This work
TRM-FL06	<i>mat A his-3+::Pccg-1-fim-1-mCherry+</i> ; <i>his-3+::Pccg-1-lifeact-egfp+</i>	This work
Oligonucleotides		
Primer	Sequence (5'-3')	
5For- <i>myo1</i>	TGAATGCGGTATTATGGCTGAG	
5Rev- <i>myo1</i>	TCTTCTGTCTCCGACTGTCTCTTTGTGCTCTC	
3For- <i>myo1</i>	TTACTGAAATCTCAACTCGTCTTTTGCTGTGC	
3Rev- <i>myo1</i>	TTATTATTGGGAGGTGGTGG	
NestFor- <i>myo1</i>	TCATATAAGACTCTCCGAGG	
NestRev- <i>myo1</i>	TTGAGGAAGAAAAGTACAGG	
Spel-Myo5 Fw	GACTAGTATGGTGACTAGCATCATCCGA	
Myo5-Pacl R	CCTTAATTAACAGTCATCTCATCATCC	
<i>hph</i> -F	GTCGGAGACAGAAGATGATATTGAAG	
<i>hph</i> -R	GTTGGTGATTTCACTAACGTTAAGTG	

FGSC-9718 by electroporation. To fuse the *sgfp* gene to the carboxyl terminus of *myo-1*, the *myo-1* gene was amplified with primers *SpeI*-Myo1-Fw and Myo1-PacI-R. The amplified and gel-purified PCR product was digested with *SpeI* and *PacI* and ligated into *SpeI*- and *PacI*-digested plasmid pCCG::C-Gly::GFP (Honda and Selker, 2009) for GFP tagging. The resulting expression plasmid pRM-FL02 (*Pccg-1-myo-1-sgfp+*) was used to transform the *his-3⁻* *N. crassa* strain FGSC9717 by electroporation.

2.3. Transformation protocols, selection of transformed strains and crosses

Transformations of *N. crassa* strains FGSC-9718 and FGSC-9717 Δ mus-51 conidia were carried out by electroporation on a Bio-Rad Gene Pulser (capacitance, 25 μ F; 1.5 kV; resistance, 600 Ω) as previously described. The heterokaryon strains selected from each transformation were crossed to the homokaryotic strains of the opposite mating type. Strains were crossed routinely on Petri plates with synthetic crossing medium (SCM) supplemented with 1% sucrose and 2% agar (Davis, 2000). To obtain the Δ myo-1 homokaryon strain, we used an microconidia isolation method (Ebbole and Sachs, 1990).

2.4. Endocytosis assays

The strains were treated as previously described (Echaui-Espinosa et al., 2012). Briefly, mycelia were grown for 12 h at 30 °C on VMM (3% agar), an agar block from the border of the colony was cut and mounted onto a coverslip and incubated at RT for 15 min. Once the coverslip was placed on the microscope and mycelia were observed, a 20 μ l drop of 2.5 μ M-FM4-64 was added at the border of the block to facilitate the diffusion of the liquid and imaged immediately on an Olympus scanning confocal microscope.

2.5. Laser scanning confocal microscopy

Strains containing fluorescent markers were grown on VMM for 12 h at 30 °C. The “inverted agar block” method (Hickey et al., 2002) was used for live-cell imaging with an inverted laser scanning microscope FV1000 FluoView™ (Olympus, Japan) equipped with a Multi-line Ar laser for excitation at 488 nm for GFP and a He-Ne(G) laser for excitation at 543 nm for mChFP. An UPlanFLN 60x, (NA 1.42) oil immersion objective was used. Time-lapse images were captured simultaneously by fluorescence and DIC

(Differential Interference Contrast) channels with FluoView™ software (Olympus, Japan). Final figures were created using Adobe Photoshop CS5 Extended (Adobe Systems Inc., San Jose, CA).

2.6. Total Internal Reflection Fluorescence Microscopy (TIRFM)

For TIRF microscopy, we used an Olympus cell[^]TIRF illuminator coupled to an IX-81 inverted microscope equipped with an APON 60xO TIRF O with Correction Collar* (NA 1.49). Images were recorded with a digital camera ORCA-Flash 2.8 (Hamamatsu Photonics K.K., Japan) for 2–3 min at 512 $\times$ 512 resolution and frame rates of 100–300 ms. CellSens Dimension software (Olympus, Japan) was used to control the camera and capture images. Final figures were created with Adobe Photoshop CS5 Extended (Adobe Systems Inc.).

3. Results

3.1. MYO-1 localization and dynamics

By protein sequence comparison (pBLAST), we identified a myosin class I homologous sequence in the genome of *N. crassa* (Galagan et al., 2003), with the locus NCU02111. The *myo-1* gene encodes a protein of 1235 amino acids with an identity of 56% compared to Myo5 of *S. cerevisiae* and 52% to Myo1 of *Schizosaccharomyces pombe*. The *N. crassa* sequence shows a structural common arrangement of a typical class I myosin (Fig. 1A) namely a globular N-terminal motor or head domain, a TH1 and SH3 domains for membrane and protein–protein interactions respectively (Fig. 1B) (Chen et al., 2012; Kim and Flavell, 2008). Growing hyphae of *N. crassa* carrying a *myo-1-GFP* allele were examined by confocal microscopy. MYO-1-GFP was found mainly as a collection of cortical patches forming a cylindrical collar (7.2 ± 1.2 μ m long; mean $\pm$ SD; $n = 25$) in the hyphal subapex; there were also scattered patches in distal parts (Fig. 2A). As the hyphae grew (Supplementary Movie S1), the collar maintained a constant distance from the apex (4.5 ± 1.0 μ m; mean $\pm$ SD; $n = 25$). MYO-1-GFP patches localized adjacent to the FM4-64-stained plasma membrane (Fig. 2C). To better visualize the structure of the MYO-1-GFP collar, we generated a 3D reconstruction from the confocal z-stack (Fig. 2D). As shown in Fig. 2E, myosin patches formed a ring around the entire circumference of the hyphal subapex.

By TIRF microscopy, we assessed the dynamics of the MYO-1 patches in the cell cortex during hyphal growth (Supplementary

Fig. 1. *myo-1* gene encodes for a putative class I myosin. (A) Predicted structure of the MYO-1, based on a Phyre2 server homology search (Kelley and Sternberg, 2009) against structure of others myosins. (B) Schematic domain structures of myosins class I from *N. crassa* MYO-1, *A. nidulans* MyoA, *S. cerevisiae* MYO5 and *S. pombe* Myo1. Parentheses show the identity of MYO-1 with the other myosins. Domains deduced from Expasy-Prositate database (<http://www.expasy.org/prosite/>).

Fig. 2. MYO-1 localization in mature hyphae. Single confocal plane images (A) MYO-1-GFP forms a subapical collar (arrows) adjacent to the plasma membrane, arrowheads show MYO-1-GFP patches in posterior regions. (B) FM4-64 staining reveals membranes and Spk localization (arrowhead). (C) Merge image of MYO-1-GFP and FM4-64 staining. (D) 3D reconstruction image of confocal z-stack showing the cortical location of MYO-1-GFP. (E) Axial view of the 3D reconstruction in (D), the yellow line indicates the cross section where the image was taken. Scale bars = 10 μm. TIRFM images of MYO-1-GFP patches during tip growth. (F) Kymograph of patch dynamics during tip growth from a 35 s video, using the Kimomaker software (Chiba et al., 2014). (G–I) Time series of individual patches lifetime. Little movement was observed in some patches before their complete internalization into the cytoplasm (I, arrow head). Time is shown in sec:msec. Scale bar = 10 μm.

Movie S2). MYO-1-GFP patches of different sizes appeared and disappeared constantly (Fig. 2F). Most of the patches displayed little or no transversal movement within the focal plane before they were internalized (Fig. 2H). However there were some instances of patches with short transversal movement prior to be internalized (Fig. 2J, arrowhead). Patch dynamics was similar in the subapex and in distal parts of the hypha; MYO-1-GFP patches internalized with an average time of $\sim 2.60 \pm 0.5$ s (mean $\pm$ SD; $n = 22$) (Fig. 2H–J).

The dynamics of MYO-1 was followed during conidial germination. Initially MYO-1-GFP appeared distributed around the cell cortex (Fig. 3A and B). As germination started, there was a near full displacement of the fluorescence to form a polar cap in the emergent tube (Fig. 3C). In young germ tubes, MYO-1-GFP patches remained accumulated in the apical dome except in the very tip (Fig. 3D and E). A robust endocytic subapical collar, the kind found in mature hyphae, was not formed until the germ tubes reached ~ 130 μm long (Fig. 3F).

3.2. Coexpression of MYO-1 with endocytosis-related ABPs

Strains expressing MYO-1-GFP together with mChFP-tagged fimbrin (FIM-mChFP) or coronin (CRN-1-mChFP) were examined. Patches of FIM-mChFP appeared next to the MYO-1-GFP patches with minimum overlapping (Fig. 4A–C). Similarly there was little overlapping of CRN-1-mChFP with MYO-1-GFP patches (Fig. 4E–G). These three proteins appeared stacked in layers with MYO-1-

GFP closer to the plasma membrane than FIM-mChFP or CRN-1-mChFP (Fig. 4D and H).

3.3. MYO-1 in septum development

MYO-1 was also involved in septum formation. Septum development was followed by staining with FM4-64. MYO-1-GFP appeared before the first sign of membrane invagination, over the developing septum on either side from the start and continued during the entire septation process (Fig. 4I–K; Supplementary Movie S3). The $\Delta myo-1$ mutant did form septa similar to those in WT.

3.4. Lack of *myo-1* alters hyphal morphology and prevents asexual and sexual reproduction

A viable strain with complete deletion of *myo-1* was generated and subjected to PCR analysis to confirm the elimination of *myo-1*. The Δ -strain grew as compact colonies (Fig. 5D) with a severe reduction in radial growth, nearly 95%, compared to the fast spreading WT strain (Fig. 5C). There was near absence of aerial mycelium and no conidiation (Fig. 5A and B).

The hyphae of $\Delta myo-1$ mutant were much slender than those of the WT strain (Fig. 5E–H, Table 2) and grew in irregular fashion with a highly uneven profile (Fig. 5H; Supplementary Movie S4). The montages of hyphal tips at consecutive times (Fig. 5I–K) showed the constancy of hyphoid shape in the WT and the

Fig. 3. MYO-1 localization during germination. Single confocal plane images showing MYO-1-GFP in (A and B) spores, (C–F) germlings up to 4 h of incubation at 30 °C. Scale bar = 10 μm.

variability of tip shape in the $\Delta myo-1$ mutant. In the latter, the profile of the growing tips deviated from a typical hyphoid with variable degrees of rounding that leaved behind a string of bulges.

3.5. Effect of *myo-1* deletion on the actin cytoskeleton and fimbrin dynamics

$\Delta myo-1$ mutant strains expressing Lifeact-GFP or FIM-GFP (see Table 1) were generated and their behavior compared with a WT strain expressing the same fluorescent markers. In the WT strain, there was a strong actin signal in the Spk and numerous cortical patches in the subapex (Fig. 6A). Some actin filaments were visible near the cortex (Fig. 6D). In the $\Delta myo-1$ mutant, actin was barely evident in the Spk, which was also not well defined by FM4-64 staining. The cortical patches in the subapex were less evident; nevertheless thick filaments were clearly obvious in the subapex occupying a larger region along the cortex as well as some visible actin filaments in internal regions within the cytoplasm (Fig. 6B). These observations were more clearly shown in TIRFM images, where we observed the absence of a well-defined collar in the subapex and the presence of larger and thicker actin cables (Fig. 6D). The actin present in the Spk showed periods of dissociation and re-association during hyphal growth (Fig. 6C). Additionally, some actin patches were displaced to the apical dome (Fig. 6C; Supplementary Movie S5).

FIM-GFP in the WT strain was concentrated in the endocytic collar and few patches were visible in posterior regions (Fig. 6E). In the $\Delta myo-1$ mutant the fluorescence signal of FIM-GFP was dispersed in the cytoplasm with only a few cortical patches that were not confined to the subapical collar as was true for the WT strain (Fig. 6F).

3.6. Endocytosis is reduced in $\Delta myo-1$ mutant

Upon addition of the lipophilic dye FM4-64, the plasma membrane was labeled almost immediately in both WT and $\Delta myo-1$

mutant strains (Fig. 7A). In the WT strain the fluorescence signal in the cytoplasm was observed first in the subapical region after 3 min and increased rapidly (Fig. 7B). In the $\Delta myo-1$ mutant, the fluorescence intensity in the cytoplasm was 14% of that in the WT after 3 min of exposure and remained low. No FM4-64 stained Spk was observed.

4. Discussion

Unlike endocytosis in yeast (Weinberg and Drubin, 2012), endocytosis in the hyphae of *Neurospora crassa* (Berepiki et al., 2011; Delgado-Alvarez et al., 2010; Echauri-Espinosa et al., 2012) and *Aspergillus* spp. (Araujo-Bazan et al., 2008; Higuchi et al., 2009; Matsuo et al., 2013; Upadhyay and Shaw, 2008) is mostly localized in a prominent place, a subapical collar several micrometers behind the apex. This endocytic collar can be seen in action in confocal videos of live hyphae tagged with Lifeact (Delgado-Alvarez et al., 2014). The images show dynamic collections of actin patches in the subapical collar. Previously, we described fimbrin, the ARP-2/3 complex (Delgado-Alvarez et al., 2010) and coronin (Echauri-Espinosa et al., 2012) as components of the endocytic patches of *N. crassa*. These three proteins are associated with late stages in the formation of endocytic vesicles. Presently, we described the occurrence of MYO-1 in *N. crassa* and its involvement in the earlier stages of endocytosis. MYO-1 is an actin nucleating factor which accumulates as cortical patches in a subapical collar. The MYO-1 collar is similar to the fimbrin and coronin collars in size and distance from the apex, but it resides closer to the plasma membrane. This stratification is likely to be related to the time of appearance of each protein and their different roles in the endocytic process.

The sequence of events we found in *N. crassa* is similar to those described in *S. cerevisiae*, where a great deal of progress has been made in elucidating details of the recruitment of proteins involved in endocytosis using live-cell fluorescence microscopy (Jonsdottir

Fig. 4. Coexpression of MYO-1 with the endocytic proteins fimbrin and coronin. Single confocal plane images showing (A–D) MYO-1-GFP with FIM-mChFP and (E–H) CRN-1-mChFP. Scale bar = 10 μ m. (D) and (H) Zoom in of the zone in the white dotted rectangles in (C) and (G). Scale bar = 2 μ m. MYO-1 localization during septum formation. Kymographic series of (I) MYO-1-GFP, (J) FM4-64 and (K) merge images of the medial focal plane during septum development.

and Li, 2004; Kaksonen et al., 2005) and time-resolved electron microscopy (Idrissi et al., 2012, 2008; Idrissi and Geli, 2014). The first step is the formation of the coat module that includes proteins such as clathrin, Sla1p, Pan1p, End3p, Sla2p (Kaksonen et al., 2005) and Ent1/2p and Yap1801/2p (Newpher et al., 2005). This coat is actin independent. In *N. crassa* the initial stages of endocytosis have not been studied. The second step in yeast (WASP/Myo module), involves the WASP homolog Las17p, the class I myosin Myo5p, and Bbc1p (Jonsdottir and Li, 2004). Myo5p arrives at the endocytosis sites just before the initiation of actin polymerization (Jonsdottir and Li, 2004; Sun et al., 2006) and last ~ 10 s, while actin and other ABPs stay associated for ~ 15 s. In *N. crassa*, the

permanence of MYO-1 is ~ 3 s and actin and ABPs is ~ 6 s (Delgado-Alvarez et al., 2010). This module remains practically motion-less at the plasma membrane and disassembles after actin polymerization stops (Kaksonen et al., 2003, 2005). We found similar dynamics of MYO-1 in *N. crassa*. The third module includes actin, Cap1/2p, Sac6p, Abp1p, and the Arp2/3 complex. All these proteins assemble into an actin-filament meshwork (Rodal et al., 2005; Young et al., 2004) that move rapidly (Huckaba et al., 2004; Idrissi and Geli, 2014; Idrissi et al., 2008; Kaksonen et al., 2003). Previously we described the dynamics of three proteins that belong to this module the Arp2/3 complex, fimbrin (Sac6p homolog) and coronin (not yet studied in yeast) where we found

Fig. 5. Effect of *myo-1* deletion on colonial growth, hyphal morphology, branching, aerial mycelium and conidiation. (A) WT and (B) $\Delta myo-1$ mutant after 48 h incubation in VMM at 30 °C. Colony morphology of (C) WT and (D) $\Delta myo-1$ mutant strains growth on VMM for 3 d at 30 °C in the dark and 2 d at 25 °C in the light. Low magnification images of the colony edge of (E) WT and (F) $\Delta myo-1$ mutant. Scale bar = 100 μ m. DIC images of hyphal phenotype of (G) WT and (H) $\Delta myo-1$ mutant. Scale bar = 20 μ m. Reconstruction of the morphological differences during hyphal growth of (I) WT and (J) $\Delta myo-1$ mutant. (K) An enlargement of the image in the white box in (J). The mutant presents temporal losses of polarized growth, leading to periods of isotropic growth. Scale bar = 10 μ m.

comparable dynamics and localization. The last module, vesicle scission and actin-driven internalization (Idrissi and Geli, 2014; Kaksonen et al., 2006) has yet to be studied in *N. crassa*.

The same ABPs found in the subapical endocytic patches are also found during septum formation. ARP-2/3 complex, fimbrin, coronin (Delgado-Alvarez et al., 2010; Echaury-Espinosa et al., 2012; Upadhyay and Shaw, 2008), and MYO-1 together with actin form scattered patches accumulated in the plasma membrane at the septation site before membrane growth and are present until

septa are formed. These patches take part of late stages of septation and seem to be part of the endocytic process that may contribute to membrane remodeling during plasma membrane growth but do not participate in the selection of the septation site or the assembly and function of the contractile actomyosin ring (Mourino-Perez, 2013; Mourino-Perez and Riquelme, 2013).

Whereas deletion of myosin-1 in *A. nidulans* (McGoldrick et al., 1995) or *A. oryzae* (Matsuo et al., 2013) proved lethal, we were able to recover a viable homokaryotic strain of *N. crassa* devoid of the

Table 2Kinetics of the WT strain and the $\Delta myo-1$ mutant.

Strain	Elongation rate ^a ($\mu\text{m min}^{-1}$)	Biomass production ^b (g d^{-1})	Hyphal diameter ^b (μm)
WT	47.63 ± 0.18	1.46 ± 0.18	11.75 ± 0.10
$\Delta myo-1$	1.89 ± 0.04	0.14 ± 0.03	5.42 ± 0.05

(mean $\pm$ standard error).^a measured in a 12 h grown mycelial at 30 °C on VMM.^b mycelia grown on liquid VMM at 30 °C for 48 h were collected, dry and lyophilized and the dye weight was compared.

Fig. 6. Influence of *myo-1* deletion on the actin dynamics in the apical and subapical regions. Single confocal plane images of hyphal tips. (A) actin localization by the reporter Lifeact-GFP in WT. (B) Lifeact-GFP in $\Delta myo-1$ mutant showing actin cables (arrow). (C) Time-series of confocal images in $\Delta myo-1$ mutant strain expressing Lifeact-GFP, white circle shows when actin in the Spk was dispersed. Time in min:sec. (D) TIRFM images in WT and $\Delta myo-1$ mutant expressing Lifeact-GFP, subapical actin patches (red arrows) and actin cables (red arrowheads). Scale bar = 10 μm . Influence of *myo-1* deletion on fimbrin dynamics. (E) Single confocal plane images of Fim-GFP in the WT, (F) Fim-GFP in the $\Delta myo-1$ mutant. Scale bar = 10 μm .

Fig. 7. $\Delta myo-1$ mutant has defects in endocytosis. (A) Comparative rates of internalization of the endocytic marker FM4-64 in the WT and $\Delta myo-1$ mutant. FM4-64 internalization monitored by confocal microscopy. Scale bar = 20 μm . (B) Graph of fluorescence intensity in the subapical cytoplasmic region (10 μm from the tip), average of pixels in 5 μm of 20 different hyphae; error bars indicate standard error.

myo-1 gene and therefore we could evaluate for the first time the phenotype of a $\Delta myo-1$ mutant. The homokaryotic $\Delta myo-1$ strain was obtained from microconidia since it was not possible to recover progeny by sexual reproduction. MYO-1 is essential for asexual or sexual reproduction, similar to $\Delta myo1$ cells of *S. pombe* that are defective in sporulation (Toya et al., 2001). As $\Delta myo-1$ mutant produces neither conidia nor ascospores, to keep the strain alive it was necessary to do mycelium transfers.

The observed disorganization of the actin cytoskeleton, caused by the absence of MYO-1, was responsible for the scant growth but also the distorted hyphal morphogenesis of the mutant. In the wild type, a fully functional actin cytoskeleton supports a robust Spk and assures a constancy of hyphoid morphology (Bartnicki-Garcia et al., 1995). Seemingly, the partly disorganized actin cytoskeleton of the $\Delta myo-1$ mutant was unable to support a steady Spk. In turn, the erratic Spk distorted the normal exocytosis pattern specified by the hyphoid model (Bartnicki-Garcia et al., 1989) creating a varying mixture of isotropic and polar growth that resulted in the observed variable bulging.

As expected, the lack of a bona fide component of the endocytic chain such as MYO-1 had a profound effect on endocytic features. The mutant was unable to form a well-defined subapical collar; there were only few actin patches along the cortex. Correspondingly, the level of FM4-64 taken up by endocytosis was drastically reduced. Similar defects in patch organization were reported in the coronin mutant (Echaurren-Espinosa et al., 2012). A conspicuous and

unusual finding of disorganization of the actin cytoskeleton was the occurrence of thick actin cables in the $\Delta myo-1$ mutant away from the cell cortex. In budding (Geli and Riezman, 1996) and fission yeast (Toya et al., 2001) deletion of myosins class I also results in disorganized actin cables. In yeast, actin assembly at endocytic places is promoted by the Arp2/3 nucleating complex (Kaksonen et al., 2003). Its activity is strongly enhanced by multiple actin nucleating promoting factors such as myosin class I. Our results show the importance of MYO-1 in maintaining the overall structure of the actin cytoskeleton of *N. crassa*. The lack of MYO-1 affects not only endocytosis but also growth and morphogenesis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fgb.2016.01.009>.

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