The fungal RNA-binding protein Rrm4 mediates long-distance transport of *ubi1* and *rho3* mRNAs

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Cytoskeletal transport promotes polar growth in filamentous fungi. In *Ustilago maydis*, the RNA-binding protein Rrm4 shuttles along microtubules and is crucial for polarity in infectious filaments. Mutations in the RNA-binding domain cause loss of function. However, it was unclear which RNAs are bound and transported. Here, we applied *in vivo* RNA binding studies and live imaging to determine the molecular function of Rrm4. This new combination revealed that Rrm4 mediates microtubule-dependent transport of distinct mRNAs encoding, for example, the ubiquitin fusion protein Ubi1 and the small G protein Rho3. These transcripts accumulate in ribonucleoprotein particles (mRNPs) that move bidirectionally along microtubules and co-localise with Rrm4. Importantly, the 3' untranslated region of *ubi1* containing a CA-rich binding site functions as zipcode during mRNA transport. Furthermore, motile mRNPs are not formed when the RNA-binding domain of Rrm4 is deleted, although the protein is still shuttling. Thus, Rrm4 constitutes an integral component of the transport machinery. We propose that microtubule-dependent mRNP trafficking is crucial for hyphal growth introducing *U. maydis* as attractive model for studying mRNA transport in higher eukaryotes.
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Introduction

Establishment and maintenance of a defined axis of polarity determines the growth and shape of filamentous fungi. Hyphae expand at the apical pole and are partitioned by septa that function as molecular barriers (Harris, 2006; Steinberg, 2007). Apical surface expansion is supported by local exocytosis of transport vesicles containing building blocks and enzymes for the synthesis of membranes and cell walls. A key element involved is the so-called

Spitzenkörper that is thought to function as a vesicle supply centre containing exocytotic as well as endocytotic vesicles (Harris, 2006; Fischer, 2007). Recently, molecular components of the polarisome such as formins that nucleate actin cables as well as components of the exocyst have been proposed to communicate with the Spitzenkörper (Harris, 2006; Fischer, 2007). In addition, microtubules are associated with the Spitzenkörper, suggesting that the long-distance transport of molecular cargos is important for polar growth (Harris, 2006; Steinberg, 2007).

The fungal pathogen *Ustilago maydis* causes corn smut disease (Feldbrügge *et al.*, 2006). A prerequisite for infection is a switch from yeast-like to filamentous growth. This morphological transition is regulated at the transcriptional level by a homeodomain transcription factor encoded at the *b* mating-type locus. The dikaryotic filament grows with a defined axis of polarity. It expands at the apical pole and inserts retraction septa at the distal pole that confine the cytoplasm to the tip compartment (Feldbrügge *et al.*, 2006).

Recently, we discovered that the RNA-binding protein Rrm4 is needed for rapid polar growth. The protein contains three N-terminal RNA recognition motifs (RRMs) and a C-terminal poly[A]-binding protein C terminus (PABC) domain predicted to function in protein–protein interaction. *rrm4Δ* strains are impaired in filament formation and pathogenicity (Becht *et al.*, 2005). The majority of *rrm4Δ* filaments grow bipolar and few retraction septa are formed indicating that Rrm4 is crucial for determining the axis of polarity (Becht *et al.*, 2006). The protein shuttles in particles along defined cytoskeletal tracks. These were identified as microtubules, as treatment with the microtubule inhibitor benomyl interfered with particle movement and bidirectionally moving Rrm4 particles co-localised with microtubules *in vivo* (Becht *et al.*, 2006). Deletion of the conventional kinesin Kin1 resulted in the formation of bipolar filaments with few retraction septa (Steinberg *et al.*, 1998). In these strains, Rrm4-containing particles accumulate at the poles, suggesting that shuttling is of functional importance (Becht *et al.*, 2006). Point mutations in the N-terminal RRM1 domain resulted in loss of function, suggesting that RNA binding is essential. *In vivo* UV cross-linking (CLIP; Ule *et al.*, 2003) revealed that Rrm4 binds RNA and that this binding increases during filamentation (Becht *et al.*, 2006). These data led to the hypothesis that Rrm4 is involved in microtubule-dependent transport of RNA (Feldbrügge *et al.*, 2008). However, it was unclear which type of RNA is bound by Rrm4 and whether the bound RNA is part of transported particles.

Here, we combine CLIP and the λN-Gfp RNA reporter system (Ule *et al.*, 2003; Daigle and Ellenberg, 2007) to show microtubule-dependent mRNA transport *in vivo*. We observe that Rrm4 binds a distinct set of mRNAs containing a potential CA-rich binding site. Target mRNAs accumulate in Rrm4-dependent ribonucleoprotein particles (mRNPs) and these particles shuttle along microtubules. Importantly, the mRNA particles co-localise with Rrm4 *in vivo*, and removal of

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RRMs in Rrm4 causes loss of motile particles, while the protein is still shuttling. Thus, Rrm4 is not simply hitchhiking but functions as integral part of the mRNA transport machinery.

Results

The poly(A)-binding protein co-localises with Rrm4 in moving particles

According to our working model, Rrm4 mediates transport of mRNA in filaments. As it is generally accepted that the poly(A)-binding protein associates with most if not all poly(A) tails of eukaryotic mRNAs (Hogan *et al*, 2008), it can be used as a molecular marker to investigate the subcellular localisation of mRNAs. Pab1 from *U. maydis* exhibits the characteristic domain structure of four RRMs in combination with a C-terminal PABC domain (um03494; 651 amino acids; MUMDB, <http://mips.gsf.de/genre/proj/ustilago/>; 47% SIMAP identity to ScPab1 from *Saccharomyces cerevisiae*, similarity matrix of proteins; Rattei *et al*, 2006). For verification of Pab1 function, we expressed fluorescently tagged versions in a strain from *S. cerevisiae* carrying the mutated allele *pab1-53^{ts}*. This strain exhibited a temperature-sensitive growth phenotype. Expression of fluorescently tagged proteins was confirmed by microscopy (Supple-

mentary Figure 1). The reduced growth rate at 28°C was complemented by expression of the poly(A)-binding protein Pab1 from *S. cerevisiae* (ScPab1) as well as by fluorescently tagged Pab1G and Pab1R from *U. maydis* (Pab1 fused at the C terminus to eGfp, enhanced version of the green fluorescent protein, Clontech, and mRfp, monomeric form of the red fluorescent protein, respectively; Campbell *et al*, 2002; Figure 1A). Thus, Pab1 from *U. maydis* functions as poly(A)-binding protein in *S. cerevisiae* and a C-terminal fusion to fluorescent proteins does not interfere with its function.

Moreover, Rrm4G (Rrm4 fused at the C terminus to eGfp; Becht *et al*, 2006) was not able to complement temperature-sensitive growth, indicating that it cannot function as poly(A)-binding protein, although it contains a similar domain architecture (three N-terminal RRMs and a C-terminal PABC domain).

To analyse the subcellular localisation of Pab1 in *U. maydis*, we generated a Pab1G-expressing strain by substituting the wild-type copy of strain AB33 by homologous recombination using an SfiI-mediated gene replacement system (Brachmann *et al*, 2004; see Materials and methods). AB33 is a derivative of wild-type strain FB2 that contains an active bW2/bE1 heterodimeric transcription factor under control of the nitrate-inducible promoter *P_{nar1}*. Thereby,

Figure 1 Pab1 and Rrm4 co-localise in shuttling particles. (A) Growth of *S. cerevisiae* strain HKY171 carrying the mutation *pab1-53^{ts}* conferring reduced growth at 28°C. Proteins expressed are indicated on the left (black triangle, 10-fold serial dilutions). (B) Filament of AB33pab1G. Rectangles indicate regions that are magnified below. Frames are taken from Supplementary Video 1 (upper part). To document motility of particles, overlays of two frames that are 0.4 s apart and coloured in red and green are shown. Arrowheads indicate moving particles (upper and lower size bar, 10 and 2 μm, respectively). (C) Filament of AB33pab1R/rrm4G. Inverted frames are taken from Supplementary Video 2 recorded with dual-colour detection. Simultaneous Gfp and Rfp images are shown juxtaposed. Red and green arrowheads mark Pab1R- and Rrm4G-containing particles, respectively. Elapsed time in seconds is indicated (size bars, 10 and 2 μm, respectively). (D) Western blots after tandem affinity purification of Rrm4GT or GT. Input fraction (after mechanical destruction of filaments) and output fraction (after TEV protease cleavage and second affinity purification step) are shown on the left and right, respectively. In the upper and lower part, proteins (given on the right) were detected using α-Gfp and α-Myc antibodies (size marker in kD on the left; *, note that α-Myc incubation detects residual amounts of Rrm4GT because of the protein A domain). (E) Filament of AB33pab1G/rrm4Δ (labelling as in B; size bars, 10 and 2 μm, respectively; frames of Supplementary Video 1, lower part).

b-dependent filamentation can be elicited by changing the nitrogen source in the medium (Brachmann *et al*, 2001; Becht *et al*, 2006).

In filaments grown for 4 h under inducing conditions, Pab1G displayed a predominantly cytoplasmic localisation reflected by negative staining of the nucleus. In addition to the dispersed cytoplasmic distribution, the fusion protein accumulated in particles that moved bidirectionally along distinct tracks within filaments (Figure 1B; Supplementary Video 1). Particle movement was inhibited in the presence of the microtubule inhibitor benomyl (50 μ M for 20 min, data not shown), indicating that these particles travelled on microtubules in a similar way as Rrm4-containing particles (Becht *et al*, 2006). To test whether Pab1 and Rrm4 accumulated in the same particles, we generated an AB33 derivative expressing Pab1R as well as Rrm4G by replacing the wild-type copy of each gene at the homologous loci. *In vivo* localisation was analysed simultaneously by recording time-lapse videos using dual-colour detection (see Materials and methods). This revealed that eGfp- and mRfp-containing particles followed the same trajectory indicative for co-localisation of Pab1R and Rrm4G in the same transport unit (97% of Rrm4G-containing particles co-localised with Pab1G-containing particles, 336 out of 347 particles, 10 videos; Figure 1C; Supplementary Video 2).

For biochemical verification, we generated an AB33 derivative expressing Rrm4GT and Pab1CM (Rrm4 fused at the C terminus to eGfp as well as Tap tag and Pab1 fused to the mRfp derivative mCherry as well as triple Myc epitope tag, respectively; Evan *et al*, 1985; Shaner *et al*, 2004; Becht *et al*, 2006). AB33GT/Pab1CM was used as control strain expressing eGfp-Tap tag under control of the *rrm4* promoter (translational fusion to 28 aa of Rrm4; see Materials and methods). After two rounds of affinity purification and TEV protease cleavage, Rrm4GCB and GCB (Gfp-calmodulin-binding domain) were detected using α -Gfp antibodies (Figure 1D, upper panel, lanes 3 and 4, respectively). Detection with α -Myc antibodies revealed that Pab1CM co-purified with Rrm4GCB but not with GCB (Figure 1D, lower panel, lanes 3 and 4, respectively).

By deleting *rrm4* in AB33pab1G, we addressed whether particle formation of Pab1G was dependent on Rrm4. Filaments grown for 4 h under inducing conditions exhibited the characteristic loss-of-function phenotype of *rrm4* Δ strains, that is, filaments failed to insert empty sections and a significant number of filaments grew bipolar (Figure 1E). Recording time-lapse videos revealed that Pab1G-containing particles were undetectable (Figure 1E; Supplementary Video 1). In addition, the cytoplasmic distribution of Pab1G was altered in *rrm4* Δ strains (Figure 1B and E). Although in AB33pab1G the fluorescence signal of Pab1G at the perinuclear region was only slightly higher compared with the growing pole, this ratio increased significantly in the absence of Rrm4 (the ratio of average fluorescence intensity in a defined perinuclear region and at the pole was 1.4 [± 0.01 , s.e.m.] in AB33pab1G versus 2.8 [± 0.09 , s.e.m.] in AB33pab1G/*rrm4* Δ ; 45 filaments analysed, $n=3$; $P<0.005$ by paired two-tailed *t*-test). This indicated that Rrm4 is required for the distribution of mRNA-binding Pab1G. Thus, Rrm4-containing particles constitute the main unit for microtubule-dependent transport of mRNAs in filaments.

Rrm4 binds distinct mRNAs *in vivo* during filamentous growth

Previously, we applied CLIP (Ule *et al*, 2003) to show that Rrm4 binds RNA *in vivo* (Becht *et al*, 2006). Here, we followed an extended CLIP protocol to identify Rrm4 targets by purification of covalently bound RNA. AB33rrm4GT filaments grown for 6 h under inducing conditions were treated with doses of UV-C irradiation that resulted in the formation of covalent bonds between RNA and protein (Ule *et al*, 2003; Becht *et al*, 2006). Rrm4GT was affinity purified and covalently bound RNA was radioactively labelled (see Materials and methods; Figure 2A). In control experiments, we omitted UV-C irradiation or applied more stringent RNase T1 treatment (Figure 2A). Bound RNA was reverse transcribed and the resulting cDNA CLIP tags were polymerase chain reaction (PCR) amplified, cloned and sequenced (see Materials and methods). Sequence analyses of two independent experiments revealed 78 unique sequences. Their length ranged from 19 to 193 nucleotides (nt) with an average of 72 nt. Comparison with the genome sequence revealed that the

Figure 2 Rrm4 binds distinct mRNAs containing a CA-rich motif. (A) CLIP analysis of AB33rrm4GT filaments. Covalently bound RNA is detected as radioactively labelled protein-RNA complexes that are larger in size than Rrm4GT (117 kD after TEV cleavage; size markers on the left; asterisk, unspecific band). UV-C irradiation and RNase T treatment is indicated above the lanes. Equal loading was verified by western blot using α -Gfp antibodies on the same membrane (bottom). (B) Graphic representation of the CA-rich consensus sequence. The height of each column represents the level of conservation, the relative height of each letter depicts its frequency at the respective position (Crooks *et al*, 2004). (C) Graph indicating significant enrichment of Rrm4 target genes (black bars) within the respective functional categories (Ruepp *et al*, 2004) relative to the frequency within the genome (white bars). One or two asterisks symbolise *P*-value of enrichment <0.05 or <0.01 , respectively (*P*-values were determined using hypergeometric distribution, Ruepp *et al*, 2004).

CLIP tags corresponded to 61 genomic loci (Supplementary Table I; MUMDB; Kämper *et al*, 2006). In six cases, we identified overlapping sequences in two subsequent experiments indicating independent cloning events. All but six genomic loci comprised predicted transcribed regions of protein-coding genes strongly suggesting that the covalently bound RNAs corresponded to mRNA (Supplementary Table I). Two CLIP tags corresponded to the 5' untranslated region (UTR), 22 to the open reading frame and 31 to the 3' UTR. In none of the cases, intron sequences were identified, whereas in three cases, sequences spanned exon–exon borders.

Applying the pattern search algorithm MEME (Bailey *et al*, 2006), a CA-rich consensus sequence was identified as the only common motif within the dataset (Figure 2B). This 21 nt motif was found in 36% of the sequences, suggesting that Rrm4 recognises CA-rich sequences. Visual inspection of the remaining mRNAs revealed that many CLIP tags carry partially similar CA-rich sequences, for example, um04628, um11097 and um1162 (Supplementary Table I), indicating the presence of degenerated motifs. Gene ontology analysis revealed a significant enrichment of the identified genes within six functional categories (Figure 2C; <http://mips.gsf.de/projects/funcat>; Ruepp *et al*, 2004). In particular, the overlapping categories 'cell-type differentiation', 'development' and 'cell fate' were consistent with a potential role of Rrm4 target mRNAs during filament formation. Sixteen (29%) of the identified genes encoded novel proteins without functional annotation.

The most prominent target sequences were derived from the *ubi1* transcript encoding a fusion protein between ubiquitin and the ribosomal protein Rpl40. We found 13 overlapping CLIP tags. The predicted transcript carried 17 direct CA repeats in its 3' UTR. Other targets that might relate to polar growth encoded for the small G protein Rho3, the septin Cdc3 and the epsin Ent1 containing an ubiquitin interaction motif (Weinzierl *et al*, 2002; Boyce *et al*, 2005). We also noted the presence of four mRNAs encoding mitochondrial proteins. In summary, Rrm4GT binds a distinct set of mRNAs *in vivo* that carry CA-rich motifs as potential binding sites.

rho3 mRNA accumulates in Rrm4-dependent particles

To analyse the subcellular localisation of potential mRNA targets of Rrm4, we performed fluorescent *in situ* hybridisation (FISH) experiments using *rho3* as example. Filaments were grown for 4 h under inducing conditions and fixed with formaldehyde. For mRNA detection, fluorescently labelled DNA oligonucleotides were directed against *egfp* or *rho3* mRNA (see Materials and methods).

In AB33, hybridisation with *rho3* probes revealed a punctuated staining of *rho3* mRNA particles throughout the cytoplasm (Figure 3A). Although the background signal was increased in the initial cell most likely because of the larger cell volume in this region, no subcellular accumulation or mRNA gradient, for example, at the poles of filaments were detected. To increase signal intensity, we generated strain AB33P_{otef}*rho3G* expressing Rho3 fused at the N terminus to eGfp under control of the strong constitutively active promoter P_{otef} (Brachmann *et al*, 2004). Quantitative PCR confirmed that mRNA amounts of *rho3* were elevated ca. 28-fold compared with AB33 (Figure 3C). Hybridisation using either *rho3* probes or a mixture of *rho3* and *egfp* probes revealed the same qualitative staining pattern as seen in AB33 (data not

Figure 3 FISH reveals accumulation of *rho3* mRNA in Rrm4-dependent particles. (A) Fixed filaments of AB33 and AB33P_{otef}*rho3G* (upper and lower part, respectively). Inverted pictures were obtained after FISH analysis with probes directed against *rho3* as well as *rho3* and *gfp* (upper and lower part, respectively). mRNA particles (filled arrowheads) and septa (asterisks) are highlighted (size bar, 10 μm). (B) Epifluorescence image of strain AB33P_{otef}*rho3G* (asterisk, septum; size bar, 10 μm). (C) Quantitative real-time PCR showing that the relative amount of *rho3* and *rho3G* mRNA is not altered in *rrm4Δ* strains (the relative amount in AB33 [*rrm4Δ*/*rho3*] was set to 1; error bars, s.d.). (D) PIA analysis of FISH-treated filaments of AB33 and AB33P_{otef}*rho3G* as well as respective *rrm4Δ* strains. Image example of AB33 analysed with *rho3* probes (inverted image labelled as in A) followed by the corresponding fluorescence intensity graph. Exemplary graphs of filaments of strains AB33*rrm4Δ* as well as AB33P_{otef}*rho3G* and AB33P_{otef}*rho3G*/*rrm4Δ* are given below. Relative fluorescence signals were plotted against the longitudinal axis of filaments (relative distance, rear pole was set to 0). Detected peaks are indicated by filled arrowheads (see Materials and methods). (E) Bar diagram of mean particle numbers determined by PIA analysis. Particles were quantified in AB33 and AB33P_{otef}*rho3G* (upper and lower part, respectively) as well as respective *rrm4Δ* strains (as labelled below; error bars, s.e.m., *n* ≥ 3).

shown or Figure 3A, respectively). As the number of mRNA particles detected strongly increased (Figure 3A), we inferred that the probes are specific. Analysing the subcellular localisation of Rho3G protein revealed that the fusion protein accumulated at retraction septa. Thus, Rho3 might have a function in septum formation (Figure 3B).

To investigate, whether the formation of *rho3* mRNA particles was dependent on Rrm4, we quantified the amount of *rho3* mRNA particles in AB33 and AB33P_{otef}*rho3G* as well as in respective *rrm4Δ* strains. For better comparison, FISH experiments were carried out investigating unipolarly growing filaments of comparable length. To enable objective detection and statistical evaluation, we quantified peaks in two-dimensional line scans derived from microscopic images using a specifically designed peak-identifying algorithm (PIA, Figure 3D; see Materials and methods, algorithm by K Zarnack, J König and M Feldbrügge to be published elsewhere).

Analysing at least 130 filaments revealed that on average 1 and 7.3 *rho3* mRNA particles were detected in AB33 and AB33P_{otef}*rho3G* ($n = 3$ and $n = 4$, respectively). In both cases, deletion of *rrm4* did not influence the amount of *rho3* mRNA (Figure 3C). Importantly, the loss of Rrm4 led to a significant reduction in particle formation (P -value < 0.05 and < 0.01 , respectively; according to paired, two-tailed t -test; Figure 3E).

In summary, FISH experiments revealed that the potential Rrm4 target mRNA *rho3* accumulated in cytoplasmic mRNA particles. Formation of particles was significantly reduced in the absence of Rrm4.

***ubi1* and *rho3* mRNAs move on microtubules within filaments**

To investigate the potential dynamic movement of Rrm4 target mRNAs, we applied RNA live imaging based on a modified version of the λ N-Gfp RNA reporter system (Daigle and Ellenberg, 2007). We used a short peptide derived from the phage protein λ N fused to triplicate eGfp repeats (λ NG³) and its 15 nt minimal recognition hairpin, termed boxB. λ NG³ was expressed under control of the arabinose-regulated promoter P_{crgl} (Figure 4A). The corresponding gene was integrated at the defined *ip* locus of strain AB33 by homologous recombination resulting in strain SB1. As first mRNA we chose the most prominent target *ubi1* and inserted 16 boxB sequences downstream of the CA-rich region in the 3' UTR (*ubi1_B*; Figure 4A).

The transgene was inserted at the homologous locus replacing the wild-type allele (see Materials and methods). Southern blot analysis of the resulting strain SB1*ubi1_B* revealed that all transformants carried multiple insertions of the construct. Apparently, transformants with single homologous insertion events exhibited a reduced fitness. A possible explanation is that the boxB repeats in the 3' UTR interfered with the expression of the most likely essential gene *ubi1*. Thus, multiple copies of the boxB-containing transcript might compensate for reduced expression.

For the analysis of particle movement *in vivo*, we recorded time-lapse videos analysing strain SB1*ubi1_B* that was grown for 7 h under filament-inducing conditions (see Materials and methods). Three distinct classes of λ NG³-labelled RNA particles were observed: (1) particles that remained stationary (termed static particles), (2) those that moved significantly, but without directionality ($< 0.75 \mu\text{m}$ in any single direction; termed stochastically moving particles) and (3) particles that moved in a distinct direction for more than $0.75 \mu\text{m}$ (termed directed particles; Figure 4B and C; Supplementary Video 3). The average

number of directed particles containing *ubi1_B* mRNA per filament was 7.1 that covered an average distance of $5.5 \mu\text{m}$ (Figure 4D; 29 filaments analysed, $n = 3$). These particles moved with an average velocity of $1.65 \mu\text{m/s}$, which was within the same range as transport of Rrm4-containing particles (Becht *et al*, 2006). In control strain SB1, hardly any directed particles were observed indicating that formation of particles was dependent on the presence of boxB binding sites (one particle in 30 filaments, $n = 3$).

Treatment with CCCP (carbonyl cyanide *m*-chlorophenyl-hydrazine; $100 \mu\text{M}$; Becht *et al*, 2006), an uncoupler of the oxidative chain that depletes the cytosolic ATP pool, revealed a drastically reduced number of directed *ubi1_B* particles indicating that these depended on active transport. Remaining particles also covered shorter distances (Figure 4D). A comparable reduction in particle number and covered distance was also observed during benomyl treatment (Figure 4D) consistent with microtubule-dependent formation and transport of particles. In contrast, motion of stochastically moving particles was not affected indicating that their movement was not driven by ATP.

For verification and comparison to our FISH data, we chose *rho3* as a second target of Rrm4. As pilot experiments revealed that *rho3_B* expression was not sufficient for detection, we generated an allele expressing *rho3_B* under control of the constitutively active promoter P_{otef} (Brachmann *et al*, 2004). The corresponding construct was either used to replace the wild-type allele by homologous recombination or to insert the transgene ectopically (strains SB1*rho3_B** or SB1*rho3_B*, respectively). The latter was important for selection of transformants that expressed *rho3_B* at comparable levels as control strains (see below). As control transcript, we inserted 16 boxB sequences in the 3' UTR of *mfa2* encoding mating pheromone $\alpha 2$ (*mfa2_B*). To address the question whether the CA-rich region might function as RNA zipcode, we inserted the CA-rich 3' UTR of *ubi1* in *mfa2_B* (Figure 4A; *mfa2_ubiB*). Both alleles were placed under control of the constitutively active promoter P_{otef} and the constructs were inserted ectopically (strains SB1*mfa2_B* and SB1*mfa2_ubiB*). To exclude an influence of expression strength on particle formation, we selected a set of six transformants (two for each boxB-containing transcript; *rho3_B*, *mfa2_B* and *mfa2_ubiB*) that expressed the transgenes at comparable levels according to northern blot experiments (data not shown).

Recording time-lapse videos of strain SB1*rho3_B** or SB1*rho3_B* after 15 h under filament inducing conditions (see Materials and methods) revealed the same three classes of particles as observed for *ubi1_B*: static, stochastically moving and directed particles (Figure 4C and E; Supplementary Video 3). However, in strains expressing the *mfa2_B* control transcript, only few directed particles were observed and these covered only short distances (Figure 4C and E; Supplementary Video 4). Importantly, *mfa2_ubiB* transcripts that carried the CA-rich 3' UTR of *ubi1* formed seven times more directed particles than control transcripts *mfa2_B*. These particles covered similar distances as *rho3_B*-containing particles (Figure 4C and E; Supplementary Video 4). These data indicate that Rrm4 targets preferentially accumulate in microtubule-dependent particles and that the CA-rich 3' UTR of *ubi1* is sufficient to promote recruitment in directed particles.

Figure 4 λN -Gfp RNA reporter system reveals microtubule-dependent transport of *ubi1* and *rho3* mRNAs. (A) Schematic representation of the constructs used for the adapted version of the λN -Gfp RNA reporter system. Top: the arabinose-regulated promoter P_{crg1} drives expression of a fusion protein consisting of the λN peptide (amino acid 2–22) fused to eGfp in triplicate (bent arrow, transcriptional start); T_{nos} , heterologous transcriptional terminator of the nopaline synthase gene from *Agrobacterium tumefaciens* (Zarnack *et al*, 2006). Bottom: constructs carry 16 copies of the λN -binding site boxB inserted in the 3' UTR (CA, CA-rich sequence; *ubi1* 3', 311 nt fragment of the *ubi1* 3' UTR; *rho3* 3', 159 nt fragment of the *rho3* 3' UTR; m, 40 nt fragment of the *mfa2* 3' UTR; Res, resistance cassette; brackets, presence of multiple copies of the transgene in *ubi1_B*-containing strains). (B) Filament of SB1ubi1_B. Inverted frames are taken from Supplementary Video 3. Rectangles indicate regions that are magnified below. Arrow in upper panel indicates a static particle (top), open arrowheads show a stochastically moving particle, and filled arrowheads point towards directed particles (lower panels). Elapsed time is given in the left corner (upper and lower size bars, 10 and 2 μm , respectively). (C) Filaments of SB1 derivatives expressing different boxB-containing mRNAs. Inverted frames are taken from Supplementary Video 2 and 3 (*ubi1_B*, *rho3_B^** and *mfa2_B*, *mfa2_ubiB*; respectively). Movement of directed particles is tracked by red arrows (dark and bright colours are used in regions of overlap). (D) Graphs representing the average particle number and the average distance covered by particles. Treatment with inhibitors benomyl and CCCP as well as deletion of *rrm4* was performed using strain SB1ubi1_B (≥ 29 filaments analysed, $n = 3$; error bars, s.e.m.; $P < 0.001$, ANOVA test compared with *ubi1_B*). (E) Graphs with labelling as in D. For strains generated by ectopic insertion of the boxB-containing transgenes (*rho3_B*, *mfa2_B*, and *mfa2_ubiB*) the results of two independent transformants were combined (> 60 filaments; $n = 6$; error bars, s.e.m.; asterisk, $P < 0.005$; by paired, two-tailed *t*-test).

ubi1 mRNA and Rrm4 co-localise in directed particles *in vivo*

To answer the question whether *ubi1* mRNA co-localised with Rrm4 in directed particles, we generated strain

SB1ubi1_B/rrm4R expressing a functional fusion protein of Rrm4 and C-terminal mRfp at the homologous locus (Becht *et al*, 2006). Filament formation was induced for at least 12 h and *in vivo* localisation was analysed simultaneously.

We observed that λ NG³-labelled *ubi1_B* mRNA and Rrm4R particles followed the same trajectory (Figure 5), indicating that *ubi1* mRNA and Rrm4 co-localised in microtubule-dependent particles *in vivo*. The number of directed *ubi1_B* particles was smaller than the number of Rrm4R particles. Thus, *ubi1* mRNA might either not be present in all Rrm4 particles or the amounts of *ubi1* mRNA in some particles are insufficient for detection. In summary, we showed that the endogenous *ubi1* target mRNA and the RNA-binding protein Rrm4 are part of the same microtubule-dependent transport unit *in vivo*.

Formation of directed particles containing *ubi1* mRNA depends on Rrm4

To investigate whether formation of the *ubi1_B* particles depended on Rrm4, we deleted *rrm4* in strain SB1ubi1_B. The resulting deletion strain exhibited the loss-of-function

Figure 5 Rrm4 and *ubi1* mRNA are part of directed mRNPs *in vivo*. Filaments of SB1ubi1_B/rrm4R 20 h (left) and 12 h (right) after induction. Inverted frames are taken from time-lapse video recorded with excitation filters on a spinning wheel. Black and white arrowheads indicate Rrm4R- and *ubi1_B* mRNA-containing (λ NG³-labelled) particles, respectively. Elapsed time in seconds is given on the right. Exposure times were 225 ms (left) and 300 ms (right). Switching excitation filters from red to green fluorescence was faster than the reciprocal switch because of the experimental setup (see Materials and methods; size bars, 10 μ m).

phenotype of *rrm4* Δ strains (Becht *et al*, 2006). Time-lapse videos of filaments grown for 7 h under inducing conditions revealed the formation of static and stochastically moving particles (Figure 6A; Supplementary Video 5). However, the number of directed *ubi1_B* particles was drastically reduced and the remaining particles covered only short distances (Figure 4D). Thus, consistent with our FISH results, Rrm4 was crucial for the formation of directed particles. We also noticed that the amount of stochastically moving particles was enriched in the region around the nucleus in comparison to the filament poles (Supplementary Video 5), indicating that the distribution of *ubi1_B* transcripts was disturbed in the absence of directed particles.

Figure 6 Rrm4 is required for the formation of directed *ubi1_B* mRNA particles. (A) Filament of SB1ubi1_B/rrm4 Δ . Inverted frames are taken from Supplementary Video 5. Rectangles indicate regions that are magnified below. Arrows show a static particle (left) and open arrowheads point towards a stochastically moving particle (right). No directed particles are detectable. Elapsed time is given on the right (upper and lower size bars, 10 and 2 μ m, respectively). (B) Filament of SB1ubi1_B/rrm4 Δ RRM4. Frames are taken from Supplementary Video 6 (upper part, labelling as in A). Rrm4^{ARRM4}-containing particles that are moving bidirectionally along microtubules are indicated by white arrowheads (note that no directed *ubi1_B* mRNA-containing particles are detectable, see Supplementary Video 6, lower part).

Rrm4 is an integral part of the *ubi1* mRNA transport machinery

For further investigation of the role of Rrm4 during mRNA transport, we genetically dissected its RNA binding function from the capacity of microtubule-dependent protein shuttling. To this end, we generated strain SB1ubi1_B/rrm4^{ARRM}R expressing an Rrm4 variant with a deletion from aa 18 to 499 resulting in the removal of all three RRM. This truncated version still contained the C-terminal PABC domain that is necessary for microtubule-dependent shuttling of Rrm4 (Becht *et al*, 2006).

Microscopic analysis of filaments grown for 7 h under inducing conditions revealed that the strain displayed the loss-of-function phenotype, reflected by an increased amount of bipolar cells and hardly any retraction septa. Although the cytoplasmic staining was slightly increased, Rrm4^{ARRM}R-containing particles still shuttled along microtubules comparable to the full-length protein (Figure 6B; Supplementary Video 6). Importantly, the number of directed *ubi1_B* particles was drastically reduced and the remaining particles covered only short distances (number of particles in SB1ubi1_B/rrm4^{ARRM}R and SB1ubi1_B/rrm4R = 0.2 ± 0.03 s.e.m. and 4.2 ± 0.3 s.e.m., respectively; covered distance $1.8 \mu\text{m} \pm 0.07$ s.e.m. and $5.2 \mu\text{m} \pm 0.1$ s.e.m.; $P < 0.01$ and 0.005 , respectively, according to paired, two-tailed *t*-test, 30 filaments per strain, $n = 3$). Reminiscent with *rrm4Δ* strains, the formation of static and stochastically moving particles was still observed (Supplementary Video 6). Hence, mRNA transport was lost, although Rrm4 was still able to shuttle along microtubules. This indicated that Rrm4 is not simply hitchhiking as an mRNA-associated component but rather constitutes an integral part of the mRNA transport machinery.

Discussion

Combining CLIP, FISH and RNA live imaging revealed that Rrm4 is the key RNA-binding protein involved in microtubule-dependent transport of distinct mRNAs. Initially, we showed that loss of Rrm4 resulted in drastically reduced formation of particles containing poly(A)-binding protein Pab1. As this type of protein binds the poly(A) tails of most if not all mRNAs (Hogan *et al*, 2008), we conclude that Rrm4-containing mRNP particles are the main mRNA transport unit in filaments. The reduction of particles correlated with defects in filamentation. Elucidating the molecular connection between long-distance transport of mRNAs and the determination of polarity will be one of the most important challenges for future experiments. At present, key RNA-binding proteins mediating mRNA transport are only known in few cases, for example, She2p in *S. cerevisiae* and Staufin in *Drosophila melanogaster* (Martin and Ephrussi, 2009).

Rrm4 recognises distinct mRNAs in vivo

Earlier attempts to discover Rrm4 targets applying SELEX and the yeast three-hybrid system failed to identify essential targets (König *et al*, 2007). Thus, we applied the CLIP technique that was established to investigate the RNA-binding capacity of mammalian splice regulator Nova (Ule *et al*, 2003). The strength of CLIP is the *in vivo* formation of irreversible covalent bonds between RNAs and the protein of interest, allowing rigorous purification schemes (Ule *et al*, 2005). The Tap tag enabled binding of the purified Rrm4

protein to solid beads for further enzymatic steps (Ule *et al*, 2005; Becht *et al*, 2006). Our CLIP analysis identified more than 50 candidate target mRNAs of Rrm4. The majority of mRNAs were only found once implying that future approaches such as high-throughput sequencing of CLIP-isolated RNAs will reveal additional target sequences (HITS-CLIP; Licatalosi *et al*, 2008; Wang *et al*, 2009; Yeo *et al*, 2009).

According to transcriptome-wide expression profiles of b-dependent filaments (J Kämper, unpublished data), the target mRNAs comprised abundant but also scarce mRNAs, for example, *ubi1* and *rho3*, respectively. *ubi1* mRNA was identified 13 times, suggesting that this highly expressed mRNA is heavily transported. Consistently, it contains an extended CA-rich region (see below).

The proteins encoded by putative Rrm4 target mRNAs were enriched in distinct functional categories implicated for example in development and cell fate. Bioinformatics revealed a CA-rich consensus sequence indicating a potential binding site of Rrm4. The assumption that CA-rich sequences are recognised by Rrm4 was strengthened by our observation that the CA-rich 3' UTR of *ubi1* functioned as zipcode during Rrm4-dependent mRNA transport. In comparable approaches analysing the mammalian splice regulators Nova and hnRNP A1, CLIP tags also contained the binding site of the respective protein (Ule *et al*, 2003; Guil and Cáceres, 2007). Interestingly, CA-containing motifs have been described earlier to mediate mRNA localisation in neurons (Andken *et al*, 2007) and function as splice enhancer elements in animals that are specifically recognised by the RRM splice regulator hnRNP L (Hui *et al*, 2005).

Target mRNAs accumulate in Rrm4-dependent particles

To validate our CLIP analysis, we established FISH and RNA live imaging. FISH is a powerful *in situ* technique that was, for example, recently applied to show that ca. 70% of all analysed genes encode subcellularly localised mRNAs in *D. melanogaster* (Lécuyer *et al*, 2007). Our approach revealed that *rho3* mRNA accumulated in cytoplasmic particles. In the absence of Rrm4, particle numbers decreased significantly, consistent with the notion that *rho3* mRNA is a target of Rrm4. Overexpression of *rho3G* mRNA also showed Rrm4-dependent particle formation. Moreover, the increased expression resulted in more mRNA molecules being incorporated into particles indicating that the amount of *rho3* mRNA in these particles is not saturated at wild-type expression levels.

Apparently, the absence of Rrm4 prevents formation of functional transport mRNPs. As the amount of *rho3* mRNA did not differ in the absence of Rrm4, this effect is most likely not because of indirect processes such as altered mRNA stability, but a direct consequence of the loss of Rrm4. Consistently, RNA live imaging indicated that this key RNA-binding protein functions as integral part of the transport machinery (see below).

Target mRNAs of Rrm4 are transported on microtubules

RNA live imaging was applied earlier to investigate actin- or microtubule-dependent movement of specific mRNAs in a variety of systems, such as fungi, higher plants, flies and mammalian cells (Bertrand *et al*, 1998; Fusco *et al*, 2003; Hamada *et al*, 2003; Weil *et al*, 2006). Recent modifications include the inducible expression of a triplicate Gfp fusion

protein (Haim *et al*, 2007) and use of a short peptide derived from λ N as heterologous RNA-binding component (Daigle and Ellenberg, 2007). In our RNA live imaging system, we used inducible expression of a fusion protein consisting of λ N and triple eGfp in combination with genomic integration of boxB-containing targets to detect endogenous mRNAs.

Using this modified version revealed that mRNAs formed three distinct classes of particles: static, stochastically moving and directed particles. The motility of the latter was dependent on active transport along microtubules. Crucially, *ubi1* mRNA and Rrm4 co-localised in these directed particles. These results are consistent with our previous *in vivo* co-localisation and inhibitor studies showing that Rrm4 moves bidirectionally along microtubules. So far, *in vivo* co-transport of an RNA-binding protein with its endogenous target mRNA was only shown for Staufen and *oscar* transcripts during oocyte development in *D. melanogaster* (Zimyanin *et al*, 2008).

Importantly, the CA-rich 3' UTR of *ubi1* was sufficient to confer an increase in frequency and length of mRNP movement. We infer that, although any mRNA seems to have the potential to be moved by this transport system, zipcode-containing Rrm4 targets are transported preferentially. Unspecific transport of bulk mRNA might function to distribute mRNA in filaments. This might also explain why distribution of the mRNA-binding protein Pab1 was disturbed in the absence of Rrm4 particles.

Genetic dissection of RNA binding and the capacity to shuttle in particles was achieved by introducing an Rrm4 variant without RNA-binding domain that still moved bidirectionally along microtubules. Crucially, the formation of directed mRNA particles was almost abolished in the respective strain. This important finding indicates that Rrm4 is not simply co-transported with mRNAs but rather constitutes an integral component of the mRNA transport machinery.

Using MS2-Gfp-based RNA live imaging, the same three classes of mRNA particles were detected in mammalian cells (Fusco *et al*, 2003). Directed particles also moved on microtubules and the presence of the β -actin mRNA zipcode augmented both the frequency and the length of mRNP movement. mRNAs without zipcodes were also transported indicating that unspecific transport might function to actively distribute mRNAs throughout the cytoplasm (Fusco *et al*, 2003). Consistently, in embryos of *D. melanogaster*, localising *hairy* as well as non-localising *krüppel* transcripts actively moved on microtubules. However, the presence of mRNA zipcodes increased frequency and duration of directed movement (Bullock *et al*, 2006).

The role of microtubule-dependent mRNP transport in *U. maydis*

One of the main functions of mRNA transport is the spatio-temporal regulation of gene expression (St Johnston, 2005; Martin and Ephrussi, 2009). Thus, microtubule-dependent transport of *rho3* mRNA might regulate local activity of Rho signalling. In accordance, we observed that Rho3 accumulates at retraction septa, at which it might function in septum formation.

In *S. cerevisiae*, *RHO3* mRNA is transported actin dependently to the daughter cell pole before local protein accumulation (Aronov *et al*, 2007). As Rho signalling regulates the

action of polarisome and exocyst (Guo *et al*, 2001; Dong *et al*, 2003), local translation could lead to local activation of these important polarity determinants. A comparable concept of activation on local translation has recently been shown for the mammalian counterpart RhoA during growth cone collapse in neurons (Wu *et al*, 2005).

For *ubi1* mRNA encoding a fusion protein of ubiquitin and the ribosomal protein Rpl40, the need for long-distance transport is less evident. At present, we can only speculate that, for example, distribution of *ubi1* mRNA is important to guarantee even proteasome-mediated degradation of cytoplasmic proteins. Rpl40 might exert extra-ribosomal functions as described for the human ribosomal protein L13a that is deposited with mRNAs to mediate transcript-specific translational regulation in the cytoplasm (Mazumder *et al*, 2003; Ray *et al*, 2007).

In principle, microtubule-dependent transport of mRNAs in *U. maydis* might support symmetric and/or asymmetric localisation of encoded proteins within filaments. This concept was initially proposed for actin-dependent mRNA transport during budding in *S. cerevisiae*. For example, mRNA transport promotes the symmetric distribution of the membrane protein Ist2p between mother and daughter cell as well as the asymmetric accumulation of the transcription factor Ash1p in the daughter cell nucleus (Jansen, 2001).

In conclusion, using a combination of CLIP, FISH and λ N-Gfp RNA reporter system, we present the first *in vivo* evidence for microtubule-dependent mRNA transport in fungi. The exquisite compatibility of the two *in vivo* approaches is exemplified by the fact that the CLIP-derived CA-rich 3' UTR of *ubi1* functioned as zipcode during λ N-Gfp-visualised mRNA transport. At present, microtubule-dependent transport is only known from higher eukaryotes and intensively studied during development, cell migration and neuronal processes (St Johnston, 2005; Lécuyer *et al*, 2007; Rodriguez *et al*, 2008). As the basic concepts appear to be conserved in fungi, flies and humans, *U. maydis* will serve as a simple model system to uncover fundamental principles of long-distance mRNA transport.

Materials and methods

Strains and growth conditions

Escherichia coli K-12 derivatives DH5 α (Bethesda Research Laboratories) and Top10 (Invitrogen) were used for cloning purposes. For complementation studies in *S. cerevisiae*, the temperature-sensitive strain HKY171 was used (*pab1::HIS3* [*pab1-53^{ts}TRP1* CEN], *ura3*, *leu2*, *trp1*, *his3*, *ade2*, *MAT α* ; Morrissey *et al*, 1999). Transformation and cultivation were performed using standard techniques. Growth conditions for *U. maydis* strains and source of antibiotics were described earlier (Brachmann *et al*, 2004). Strains were constructed by transformation of progenitor strains with linearised plasmids (Supplementary Table II). All homologous integration events were verified by Southern blot analysis (Brachmann *et al*, 2004). Filamentous growth of AB33 variants was induced by shifting 20, 50 or 150 ml of exponentially growing cells ($OD_{600} = 0.4-0.5$) from complete medium (CM) to nitrate minimal medium (NM) each supplemented with 1% glucose (glc) unless otherwise noted. Cells were incubated at 28°C shaking at 200 r.p.m. AB33pab1G, AB33pab1R/rrm4G and AB33pab1G/rrm4 Δ were induced for 4 h. For FISH analysis, strains AB33, AB33rrm4 Δ , AB33P_{otef}rho3G and AB33P_{otef}rho3G/rrm4 Δ were grown in CM (1% glc) to an $OD_{600} = 0.35$ at 28°C and 200 r.p.m. Filaments were induced by shifting the medium to NM (1% glc) and additional growth for 4 h. For live imaging of RNA, the duration of filament induction was

adjusted to compensate for varying expression levels of boxB-containing target mRNAs. Strains SB1, SB1ubi1_B and SB1ubi1_B/rrm4Δ were grown in CM medium containing 1% arabinose (ara) to elicit P_{crg1} -mediated expression of λ NG³ and shifted to NM medium with 1% ara to induce filament formation. Filaments were induced for 7 h before microscopic analysis. Strains SB1rho3_B*, SB1rho3_B, SB1mfa2_B and SB1mfa2_ubiB were grown in CM (1% glc) and shifted to NM (1% ara) 15 h before microscopic analysis. Strain SB1ubi1_B/rrm4R was shifted comparably and analysed after 12 and 24 h.

Plasmids and plasmid constructions

Standard molecular techniques were followed. Plasmids pCR2.1-Topo (Invitrogen) and pBluescriptSKII (Stratagene) were used as cloning vehicles. Genomic DNA of wild-type strain UM521 (*alb1*) was used as template for PCR amplifications unless otherwise noted. Detailed plasmid description, constructions and oligonucleotide sequences (Supplementary Table III) are given in Supplementary Data. All constructions were confirmed by sequencing. Plasmid sequences are available on request.

CLIP experiments

CLIP experiments were performed as described earlier (Becht *et al*, 2006) including the following steps. After resuspension in 80 µl CB-PNK buffer, the first linker was ligated over night at 16°C with shaking intervals as described. The reaction contained 60 U T4 RNA ligase 1 (NEB), 40 U RNasin, 10 µl 10 mM ATP and 5 µl kinase-treated RNA oligonucleotide oR043802 (20 pM/µl; 5'GAUCUGAGC CUGGAGCU3'; modified at the 3' end with fluorescein). Beads were washed three times with 1 ml CB-PNK buffer and resuspended in 80 µl CB-PNK buffer.

After membrane transfer, the RNA was prepared as follows. 1–2 mm membrane slices containing Rrm4 cross-linked to RNA were excised. A measure of 200 µl PK1 (100 mM Tris-HCl, pH 7.5; 50 mM NaCl; 10 mM EDTA; 10 U/ml proteinase K, Roche; pre-incubated for 20 min at 37°C) was added before incubating for 20 min at 37°C shaking at 1000 r.p.m. A measure of 200 µl PK2 (100 mM Tris-HCl, pH 7.5; 50 mM NaCl; 10 mM EDTA; 7 M urea) was added followed by 20 min incubation as before. Addition of 530 µl AE-phenol/chloroform (pH 5.3) was followed by 20 min incubation as before. RNA was precipitated by adding 50 µl 3 M sodium acetate (pH 5.3), 0.5 µl glycogen (5 mg/ml, Ambion), 500 µl ethanol and 500 µl isopropanol followed by incubation at –20°C over night. RNA was pelleted by centrifugation for 10 min at 45 000 g and washed with 1 ml 70% ethanol. Pellet was resuspended in 20 µl H₂O. For second linker ligation, 3 µl 10 mM ATP, 3 µl 10 × T4 RNA ligase 1 buffer (NEB), 2 µl RNA oligonucleotide oR043801 (40 pM/µl; 5'GCAGUUGAUGUUUUUCUGAUGC3'), 1.5 µl T4 RNA ligase 1 (NEB) and 0.5 µl RNasin were added followed by over night incubation at 16°C. DNA was removed by adding 53 µl H₂O, 10 µl 10 × DNase I buffer (Roche), 2 µl RNasin and 5 µl 10 U/µl DNase I (Roche) before incubating for 20 min at 37°C. A measure of 300 µl H₂O and 400 µl AE-phenol/chloroform (pH 5.3) was added followed by 30 s of shaking at 1400 r.p.m and centrifugation for 5 min at 16 000 g. RNA was precipitated by adding 50 µl 3 M sodium acetate (pH 5.3), 1 µl glycogen, 500 µl ethanol and 500 µl isopropanol followed by incubation at –20°C over night. RNA was pelleted by centrifugation for 10 min at 50 000 g and washed with 1 ml 70% ethanol. Pellet was resuspended in 10 µl H₂O. For reverse transcription, 2 µl 10 × buffer RT (Qiagen), 2 µl dNTP mix (Qiagen), 2 µl 10 µM oMF970 (see Supplementary Table III), 1 µl RNasin (1:4 dilution), 1 µl Sensiscript S RT (Qiagen) and 2 µl H₂O were added followed by incubation at 37°C for 60 min. Resulting cDNA was amplified by standard PCR using oligonucleotides oMF970 and oMF973 (see Supplementary Table III). PCR products ranging from 100 to 400 bp were purified using agarose gel electrophoresis. The DNA was subjected to a second round of PCR using oMF969 as well as a mixture of oMF974, oMF975 and oMF976 (see Supplementary Table III) reducing primer dimer amplification. Resulting products were purified using agarose gel electrophoresis, cloned and sequenced.

Tandem affinity purification and quantitative real-time PCR

Experiments were carried out as described in Supplementary data.

Fluorescence in situ hybridisation

For FISH analysis, a protocol was adapted from Long *et al* (1995). A detailed description is given in Supplementary data.

Microscopy, image processing and quantitative analysis

Cell suspensions were dropped on glass slides covered by a thin layer of agarose (2% w/v) and analysed using a Zeiss Axioplan II microscope equipped with objective lenses Plan Neofluor and Plan Apochromat (both 100-fold magnification; numerical aperture 1.3 and 1.4, respectively). Epifluorescence was observed using Gfp (ET470/40BP, ET495LP, BP525/50) and TexasRed (HC562/40BP, HC593LP, HC624/40BP) filter sets. To detect Cy3 either TexasRed or TRITC (HC543/22BP, HC562LP, HC593/40BP) filter sets were used. Filters were obtained from AHF Analysentechnik. Frames were taken with a cooled CCD camera (CoolSNAP HQ, Photometrics). Microscope and camera were controlled by MetaMorph 7.5 (Molecular Devices). The same software was used for measurements and image processing including adjustment of brightness, contrast and γ values as well as correction of background unevenness. Particle intensity was enhanced by applying camera binning of 2 × 2. For co-localisation studies of *ubi1* mRNA and Rrm4 time-lapse videos were recorded using a filter wheel (Visitron Systems) equipped with excitation filters for Gfp (S480/25) and Rfp (S565/25). Filter switch and detection of fluorescence were controlled using the multidimensional acquisition mode. The settings were optimised to avoid bleed through of the fluorescence signal resulting in intervals of 225 (or 300) ms and 670 (or 705) ms for the switch from Rfp to Gfp and Gfp to Rfp, respectively. This was controlled by recording time-lapse videos with mixtures of strains that expressed either eGfp or mRfp fusion proteins. Exposure time was 225 (or 300) ms. Emission was detected using a dual-band filter (Gfp: FT 480, BP503-545; Rfp: FT565, BP591-647).

For simultaneous dual-colour detection, the dual-view device (MAG Biosystems) was used. A dichroic beamsplitter and a filter designed for Gfp and Rfp (eGfp/mRfp; Chroma) were used to simultaneously excite the respective fluorophores. A second dichroic beamsplitter (dcxr565) within the dual-view device splitted the fluorescent signals into the respective colours, which were separately filtered with emission filters (Gfp HQ510/30; Rfp HQ650/75), before the different channels were simultaneously imaged on different regions of the CCD chip.

For quantification of mRNA particles, time-lapse videos (80 frames, 150 ms exposure time) were recorded for at least 29 filaments per strain in three independent experiments. Particles were tracked manually. Those that travelled more than 0.75 µm in a single direction were classified as directed particles. For strains carrying ectopic insertion of a boxB-containing transgene, data obtained from two independent transformants were combined. To quantify Pab1G signals in filaments, the average fluorescence intensity at the hyphal tip and at the perinuclear region was determined using a mask of 1.67 µm². Forty-five filaments were analysed for each strain in three independent experiments.

Inhibitor studies

A measure of 20 ml of cell suspension were incubated in the presence of either 100 µM CCCP (Sigma-Aldrich) or 50 µM benomyl (Sigma-Aldrich). Samples were incubated for 15–30 min at 28°C with agitation followed by microscopic analysis (Becht *et al*, 2006).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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