Endosomal transport of septin mRNA and protein indicates local translation on endosomes and is required for correct septin filamentation

Sebastian Baumann^{1,2}, Julian König^{2,†}, Janine Koepke^{2,‡} & Michael Feldbrügge^{1,2,*}

Abstract

Endosomes transport lipids and proteins over long distances by shuttling along microtubules. They also carry mRNAs on their surface, but the precise molecular function of this trafficking process is unknown. By live cell imaging of polarized fungal hyphae, we show microtubule-dependent transport of septin mRNA and encoded septin protein on the same shuttling endosomes. Consistent with the hypothesis that septin mRNA is translated on endosomes, the accumulation of septin protein on endosomes requires the recruitment of septin mRNA. Furthermore, ribosomal proteins co-localise with shuttling endosomes, but only if mRNA is present. Importantly, endosomal trafficking is essential for an efficient delivery of septin protein to filaments at growth poles, a process necessary to establish unipolar growth. Thus, we propose that local mRNA translation loads endosomes with septins for assembly and efficient delivery to septin filaments.

Keywords endosome; membrane trafficking; RNA live imaging; RNA transport; septin

Subject Categories RNA Biology; Membrane and Intracellular Transport

DOI 10.1002/embr.201338037 | Received 26 September 2013 | Revised 10 October 2013 | Accepted 14 October 2013 | Published online 15 December 2013

Introduction

A wide-spread mechanism for intracellular long-distance trafficking is the microtubule-dependent transport of endosomes. These function as carrier platforms for membrane lipids and proteins [1]. Particularly in highly polarized cells, endosomes shuttle extensively along microtubules to coordinate antero- and retrograde trafficking [2]. In addition to the typical cargos, endosomes are important for the transport and subcellular localisation of mRNAs [1,3].

Cytoskeletal transport of mRNAs is commonly assumed to position mRNAs within the cell for their local translation, an important mechanism to mediate spatial gene expression [4,5]. Key components of the transport machinery are sequence-specific RNA-binding proteins that act in concert with other RNA-binding proteins, such as the poly(A)-binding protein, to form large complexes called messenger ribonucleoproteins (mRNPs). These mRNPs are connected to molecular motors via adaptor proteins for transport along the cytoskeleton. mRNA translation commonly occurs upon its deposition at the final destination [6]. However, it is also often observed that mRNPs shuttle extensively back and forth without recognisable deposit and the precise molecular reason for this remains elusive [4].

In *Ustilago maydis* a morphological switch from yeast cells to unipolarly growing hyphae is a prerequisite for pathogenicity. The formation of these highly polarized cells depends on microtubule-dependent mRNA transport. In the absence of functional microtubules or molecular motors such as kinesins and dynein, hyphae grow bipolar and the insertion of retraction septa is disturbed [7]. A crucial component in this process is the RNA-binding protein Rrm4, which is responsible for the transport of mRNAs such as *ubi1* and *rho3* encoding a natural ubiquitin fusion protein and a small G protein, respectively (supplementary Movie S1, [8–10]). However, transported mRNAs do not accumulate at distinct subcellular sites. Thus, the precise role of mRNA transport is still unclear [9]. Recently, we discovered that mRNAs are co-transported with Rrm4-positive endosomes shuttling along microtubules [3]. Importantly, loss of Rrm4 does not interfere with endosomal movement, but endosomes lacking mRNAs appear to be disturbed in promoting polar growth [3].

In this study we focused on the septin Cdc3 [11,12] because its mRNA constituted an *in vivo* target of Rrm4 in previous CLIP experiments [9]. Septins are ubiquitous guanosine triphosphatases that assemble into higher-order structures such as rings and filaments [13]. Thus, the septin Cdc3 was a prime candidate to link Rrm4 function to morphological defects of corresponding mutants.

1 Institute for Microbiology, Cluster of Excellence on Plant Sciences, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

2 Department of Organismic Interactions, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany

*Corresponding author. Tel: +49 (211) 81 15475; Fax: +49 (211) 81 15370; E-mail: feldbrue@hhu.de

† Present address: Laboratory of Molecular Biology, Medical Research Council, Cambridge, UK

‡ Present address: Biomedical Research Centre, Pneumology, Philipps University Marburg, Marburg, Germany

Results and Discussion

Cdc3 is important during establishment of unipolar growth

To investigate the role of Cdc3 in cell polarity we analysed deletion strains in the genetic background of strain AB33 (supplementary Table SI–II) in which unipolar hyphal growth can be elicited by switching the nitrogen source. Hyphae grow with a defined axis of polarity expanding at the growth pole and insert retraction septa at the basal pole (supplementary Fig S1A). Four hours post induction (h.p.i.), a significant number of *cdc3Δ* hyphae grew bipolar, lacking retraction septa reminiscent of defects observed in *rrm4Δ* strains (Fig 1A and B; [11]). However, the majority of *cdc3Δ* cells switched to correct unipolar growth and also formed septa after prolonged induction (6 and 9 h p.i.; supplementary Fig S1B–C). To further investigate these temporal morphology differences, hyphae were treated with calcofluor-white that stains chitin at growth poles and retraction septa. Remarkably, only one growth pole was predominantly stained in bipolar *rrm4Δ* hyphae (supplementary Fig S1D), indicating that these initially elongate at one pole and subsequently switch growth direction to form an alternative second growth pole. Consistently, the number of bipolar *rrm4Δ* hyphae was increasing over time (supplementary Fig S1C). In contrast, bipolar *cdc3Δ* hyphae are able to switch to correct unipolar growth at later points in time (supplementary Fig S1B–C). In comparison to each single deletion mutant the *rrm4Δ/cdc3Δ* double mutant was more severely affected in unipolar growth and septum formation (Fig 1A and B; supplementary Fig S1B–C), indicating that both Rrm4-mediated mRNA transport and correct septin function are crucial for efficient unipolar growth.

Rrm4 mediates microtubule-dependent transport of septin *cdc3* mRNA

Next, we addressed whether *cdc3* mRNA is a target of the Rrm4-dependent mRNA transport machinery. Previously, we have shown that Rrm4 co-localises with distinct endosomes that can be stained with the lipophilic red-fluorescent dye FM4-64, are positive for the SNARE Yip1 and transported by Kin3 and split dynein [3]. To quantify endosomal localisation of Rrm4 we analysed the dynamic co-localisation in hyphae expressing Rrm4G and the small G protein Rab5aC N-terminally fused to mCherry (see Materials and Methods). Rab5aC served as a marker for this endosomal compartment (supplementary Fig S2A, Movie S2; [14]) and localised to distinct shuttling endosomes independent of Rrm4 (supplementary Fig S2B, Movie S2; [3]).

We detected 48 ($\pm$ 8) Rrm4G-positive signals on shuttling units per hyphae (668 Rrm4G signals in 14 hyphae). To reduce background staining of Rab5aC (supplementary Fig S2C), hyphal tips were bleached and immediately analysed via millisecond alternating laser excitation microscopy (msALEX). 81% of bidirectionally moving Rrm4G and Rab5aC signals co-localised, indicating the presence on identical shuttling endosomes (supplementary Fig S2C; 126 endosomes in 6 hyphae). Occasionally, when only Rrm4G signals were observed it was unclear whether the Rab5aC signal was below the detection limit, resulting in a potential underestimation of co-localising signals. In fact, since Rrm4G did not stain additional cytoplasmic structures (supplementary Movies S1 and S2) it can be considered a specific marker for this endosomal compartment [3].

To study *cdc3* mRNA transport we applied RNA live imaging [9]. For mRNA visualisation, a modified λ N*G² peptide (supplementary Fig S2D) fused to double Gfp (λ N*G²) was expressed under control of an arabinose-inducible promoter and 16 copies of the corresponding *boxB* binding sites were inserted in the 3' UTR of *cdc3* (*cdc3B*¹⁶; Fig 1C, see Methods). This set-up for the first time allowed detection of transported mRNAs at endogenous expression levels (Fig 1D; supplementary Movie S3). Comparable to previous results [9], we observed three types of particles: static ones, particles with short and restricted movement as well as particles that moved in a distinct direction, termed directed particles (supplementary Movie S3). The latter were particularly interesting, because their movement resembled those of target mRNAs co-localising with Rrm4 [9]. In contrast, hyphae lacking *boxB*¹⁶-containing mRNAs did not show directed movement of λ N*G² (13 hyphae analysed).

About one directed *cdc3B*¹⁶ mRNA particle was present per hypha (Fig 1E). No significant difference in velocity or directionality was determined comparing mRNAs moving towards the growth pole or the septum of hyphae (Fig 1E). The range of directed movement was from 2.4 to 38 μ m (Fig 1E). In some cases, particles exhibiting restricted movement started to move directed and *vice versa* (supplementary Fig S2E), likely resembling on- and offloading of mRNPs from the transport machinery. Loss of directed movement occurred all along the hypha, however, we did not specifically observe such potential offloading events at the growth pole or an accumulation of *cdc3B*¹⁶ mRNA at the hyphal tip (Fig 1D, supplementary Movie S3).

Deletion of *rrm4* resulted in the loss of directed particles leaving only immobile or restricted moving *cdc3B*¹⁶ mRNAs (Fig 1E and F; supplementary Movie S4). Importantly, the absence of Rrm4 resulted in a decrease of neither *cdc3* mRNA nor of protein amounts (supplementary Fig S2F–G).

To address whether *cdc3B*¹⁶ mRNAs co-localise with Rrm4-positive endosomes, Rrm4C (fusion to mCherry) was expressed in strains expressing λ N*G² and *cdc3B*¹⁶. Recording real time movies via msALEX revealed that *cdc3B*¹⁶ mRNAs co-localised *in vivo* with Rrm4C on shuttling endosomes (Fig 1G). Thus, *cdc3* mRNA is a target of the Rrm4-dependent mRNA transport machinery.

Cdc3 localises on shuttling endosomes in the presence of Rrm4

To find out whether Rrm4-dependent transport of *cdc3* mRNA determines the correct subcellular localisation of the corresponding Cdc3 protein, we analysed a functional N-terminal Gfp fusion (Cdc3G; Fig 1A and B). This confirmed the previously described septin accumulations at distinct subcellular sites such as septa and filaments (supplementary Fig S3B, Movie S5, [11,12]). Furthermore, the Cdc3G signal was stronger in filaments at growth poles than in the rest of the hyphae (Fig 2A; supplementary Fig S3A). Notably, this unequal distribution was abolished by treatment with the microtubule inhibitor benomyl and not detectable in *rrm4Δ* mutants (Fig 2A and B). Instead, in both cases Cdc3G formed small circles (< 1 μ m in diameter; Fig 2A and B), known from the septin Cdc10 in cells grown under suboptimal conditions [12]. This suggests that although septin filaments can be formed in the absence of Rrm4, Rrm4-dependent microtubule transport is essential for correct septin filamentation, particularly at hyphal tips.

Intriguingly, Cdc3G exhibited an additional, yet unknown localisation on shuttling units that resembled Rrm4-positive endosomes

Figure 1. Rrm4 transports *cdc3* mRNA along microtubules.

- A** Hyphae of AB33 derivatives 4 h after induction of polar growth (DIC images; size bar, 10 μ m).
- B** Percentages of hyphae after 4, 6 and 9 h p.i.. Unipolarity, bipolarity and septum formation was quantified (error bars, s.e.m.; $n = 3$ independent experiments, 2422 filaments were counted in total; note that septum formation is given relative to the values of unipolar or bipolar hyphae set to 100%).
- C** Components of the modified λN^* RNA reporter system (P_{crg1} , arabinose-regulated promoter; T_{nos} , heterologous transcriptional terminator; *cdc3B*¹⁶ carries 16 copies of boxB hairpin in its 3' UTR).
- D** Hyphal tip of a strain expressing the λN^*G^2 protein and *cdc3B*¹⁶ mRNA. Micrograph (size bar, 10 μ m) and the corresponding kymograph show directed particles (arrowheads; supplementary Movie S3).
- E** Velocity (total directed particles and those moving towards growth pole or septum; error bars represent s.e.m.; one way ANOVA, $P > 0.05$); directionality of particles (error bars represent s.e.m.; paired two-tailed t-test, $P > 0.05$); range of directed movement (whisker diagram showing median, min/max values and 25/75 percentiles); number of directed mRNPs in 100 μ m of hyphae ($n = 3$, 69 particles in 97 wt hyphae and 0 particles in 37 *rrm4Δ* hyphae; note, length of hyphae varies in tested strains; error bars represent s.e.m.).
- F** Micrograph (size bar, 10 μ m) and kymographs (bottom) showing restricted moving and static particles (arrowheads and arrow, respectively; supplementary Movie S4).
- G** Kymographs of λN^*G^2 -labelled *cdc3B*¹⁶ mRNA (green) and Rrm4C (red). Co-localisation (yellow) is indicated by arrowheads.

Figure 2. Rrm4-dependent accumulation of Cdc3G in septin filaments and on shuttling endosomes.

- A Micrographs of Cdc3G-expressing hyphae (z-plane 0 or maximum projection of z-stacks with 0.25 μ m steps; size bar, 10 μ m). Hypha at the bottom was treated with benomyl for 1 h (arrowheads mark small circle; see inset, size bar, 1 μ m).
- B Bipolarly growing *cdc3G/rrm4Δ* hypha (size bars 10, 1 and 5 μ m for top, bottom left and bottom right, respectively).
- C Kymographs of Cdc3G expressing hypha (asterisk marks septum; top untreated and bottom treated with benomyl).
- D Micrograph (top) and corresponding kymograph (bottom) of *cdc3G/rrm4Δ* hypha (size bar, 10 μ m; supplementary Movie S7).
- E Kymographs showing co-localisation of Cdc3G and Rrm4C (arrowheads).

(Fig 2C; supplementary Movie S6). Bidirectional movement of Cdc3G was inhibited by benomyl (Fig 2C) and was no longer detectable in *rrm4Δ* mutants (Fig 2D, supplementary Movie S7). In contrast, in budding cells Cdc3G was rarely observed on shuttling units (Five events in 80 cells; supplementary Fig S3C).

For co-localisation studies the signal of Cdc3G was increased using a strong, constitutively active promoter (P_{otef} , supplementary Table S1). This did not alter hyphal morphology. Co-staining with the red-fluorescent dye FM4-64, which marks Rab5a- and Rrm4-positive endosomes [3,15], revealed that Cdc3G is associated with this specific endosomal compartment (supplementary Fig S3E). Consistently, Cdc3G co-localised almost exclusively with Rrm4-positive endosomes (Fig 2E; 97% of endosomes contained both proteins, supplementary Movie S8). Taken together, we observed two specific subcellular locations of septin Cdc3G which are dependent on Rrm4: in a filament gradient at the growth pole and on shuttling Rrm4-positive endosomes.

mRNA transport is crucial for endosomal localisation of Cdc3

We observed that *cdc3* mRNA and protein localise to the same endosomal compartment (Figs 1G and 2E). In other systems, co-localisation of mRNAs and encoded proteins occur at the site of translation. Importantly, when mRNAs mislocalise the translation products also alter their location [6]. To investigate whether transla-

tion of *cdc3* mRNA takes place on Rrm4-positive endosomes, it was critical to show dynamic co-localisation of mRNA and protein at the same endosome. Thus, we overexpressed *cdc3CB¹⁶* mRNA with the constitutively active P_{otef} promoter at the endogenous locus encoding the mCherry fusion Cdc3C and carrying 16 λ N-binding sites in its 3' UTR (Fig 3A). Live cell imaging confirmed that λ N*G²-labelled *cdc3CB¹⁶* mRNAs indeed co-localised with Cdc3C on the same shuttling endosomes (Fig 3A).

However, Cdc3G protein was present at numerous endosomes, whereas *cdc3B¹⁶* mRNA is detectable at only a few of them (Figs 1D, 2E and 3A). To elaborate this we performed experiments with Rrm4 fused to triple photo-activatable Gfp (Rrm4Gp³). We chose Rrm4Gp³ as endosomal marker for these experiments because the signal intensity of Cdc3Gp³ on endosomes was too low. Photoactivation of Rrm4Gp³ visualised highly dynamic and extensive fission of Rrm4Gp³-positive endosomes (supplementary Fig S3D, at least four fission events in 24 s) indicating an intensive exchange of membrane-associated cargo. Thus, we propose that local translation of *cdc3* mRNA is needed for the entry of the encoded protein into this specific endosomal compartment and the observed distribution of translated Cdc3 throughout the entire endosomal compartment can be achieved by fusion and fission events.

We then tested whether the Rrm4-dependent localisation of *cdc3* mRNA is a prerequisite for the presence of Cdc3G protein on

Figure 3. Endosomal localisation of Cdc3G depends on presence of its mRNA.

- A** Top: schematic representation of the construct expressing Cdc3CB¹⁶ (constitutively active *P_{otef}* promoter; Cdc3 fused to mCherry and 16 boxB binding sites in the 3' UTR). Bottom: kymographs of movies recorded in strain expressing Cdc3CB¹⁶. Arrowheads indicate co-localising signals.
- B** Micrographs and corresponding kymographs depicting bidirectional movement of Rrm4C and Pab1G.
- C** Movement of Pab1G and Rrm4R^{mR1} fused to mRfp shown as in panel (B). Arrowheads indicate residual movement of Pab1G.
- D, E** Kymographs depicting λN*G²-labelled *cdc3B*¹⁶ mRNA (D) or Cdc3G protein (E). Strains carry the allele *rrm4R*^{mR1}. Arrowheads highlight movement of *cdc3B*¹⁶ mRNAs.
- F** Movement of Pab1G and Rrm4R^{RRMΔ} (Rrm4 with N-terminal deletion of three RRM domains fused to mRfp) shown as in panel (B).
- G, H** Kymographs depicting λN*G²-labelled *cdc3B*¹⁶ mRNA (G) or Cdc3G protein (H). Strains carry the allele *rrm4R*^{RRMΔ}. Arrowheads as in panel (D).

Data information: Note that kymographs shown in panel (B-C) and (F) were obtained from movies recorded sequentially in identical hyphae to ensure highest sensitivity (size bars, 10 μm).

endosomes. To this end we made use of two characterised Rrm4 mutants. Rrm4^{mR1} carries a four amino acid block mutation in RNP1 of RRM1 [3]. This mutation does not interfere with the endosomal localisation of Rrm4 (Fig 3B and C) but affects mRNA binding [8] as visualised by the subcellular localisation of the poly (A)-binding protein Pab1 [3]. In wild-type cells Pab1G shuttles extensively throughout the whole hypha without subcellular accumulation (Fig 3B). In strains expressing Rrm4^{mR1} shuttling of Pab1G is drastically reduced (Fig 3C).

Analysing *cdc3* mRNA transport in this mutant background revealed that endosomal transport of *cdc3B*¹⁶ mRNA was drastically reduced and the maximal run length was lower (Fig 3D and supplementary Fig S3F). Importantly, the reduced amount of shuttling *cdc3B*¹⁶ mRNAs correlated with the loss of Cdc3G protein on shuttling endosomes (Fig 3E), suggesting that a certain threshold of mRNA is needed to load the septin on endosomes (see below).

The second mutant, Rrm4^{RRMA}, lacks all three N-terminal RRM s [3]. This Rrm4 version still shuttled on endosomes (Fig 3B and 3F) confirming that Rrm4 is an integral part of the transport machinery and not hitchhiking on mRNAs [3]. As expected, Pab1G-labelled mRNAs were no longer detectable on Rrm4-positive endosomes (Fig 3F). Endosomal co-transport of *cdc3B*¹⁶ mRNA was almost abolished (Fig 3G and supplementary Fig S3F). Consistent with results obtained in *rrm4Δ* strains (Fig 2D)

shuttling of Cdc3G protein on endosomes was lost (Fig 3H). Taken together, *cdc3* mRNA and protein co-localise on the same endosome and the presence of *cdc3* mRNA is crucial for the entry of the encoded protein into this specific Rrm4-positive endosomal compartment. This strongly suggests that local translation on endosomes takes place.

Ribosomal proteins co-localise with shuttling Rrm4-positive endosomes

As an additional line of evidence for endosome-coupled translation we studied ectopically expressed C-terminal Gfp fusions of ribosomal proteins originating from both subunits. Based on structural predictions and previous work performed in *Saccharomyces cerevisiae* the ribosomal proteins Rps2, Rps19 and Rpl25 were selected (supplementary Table SI). All three proteins predominantly localised in the cytoplasm with a faint accumulation in the nucleus or nucleolus (Fig 4A). Moreover, shuttling of all three ribosomal proteins with Rrm4-positive endosomes was detectable after local photobleaching (Fig 4A and B; supplementary Movie S9). Closer inspection of Rps2G revealed that shuttling was lost in the absence of Rrm4 (Fig 4C). Thus, Rrm4 is not only required for the recruitment of the poly(A)-binding protein Pab1 to endosomes (Fig 3F, supplementary Movie S1, [9]) but also essential for endosomal localisation of ribosomal proteins, *cdc3* mRNA and Cdc3 protein.

Figure 4. Shuttling of ribosomal proteins on Rrm4-positive endosomes is dependent on mRNA.

A Top: micrograph before and after (upper and lower micrograph) photobleaching of Gfp fluorescence (size bar, 10 μm; asterisk indicates nucleus). Bottom: kymograph of supplementary Movie S9 (after photobleaching).

B Co-localisation (yellow) of Rrm4 (red) and ribosomal proteins (green) on shuttling endosomes.

C Shuttling of Rps2G is Rrm4-dependent.

Data information: Kymographs (in B and C) of movies recorded after photobleaching.

Rrm4 function is essential for correct formation of Cdc3 filaments

To study the function of endosomal septin transport we investigated whether endosomes may transport Cdc3 along microtubules and deposit it for assembly in filaments at growth poles. Hyphae expressing Cdc3G were bleached at the growth pole and fluorescence recovery was determined over time (Fig 5A, supplementary Fig S4A and B, Movie S10). Note that, according to the experimental set-up, hyphae were analysed for a prolonged time under the coverslip such that the Cdc3G gradient did not recover to the same extent than under optimal growth conditions (Fig 2A).

The half time of recovery ($t_{1/2}$) was 6 min (Fig 5B and C), comparable to previous studies on septin rings in *S. cerevisiae* [16] and consistent with a dynamic assembly process (Fig 5B and C, supplementary Fig S4A–B, [17]), suggesting that Cdc3G is indeed incorporated into septin filaments at the hyphal growth pole.

In hyphae treated with benomyl, $t_{1/2}$ was substantially increased to 12 min (Fig 5B, supplementary Fig S4A). This indicates that although microtubule-dependent transport is not essential for filament formation, this cellular transport process is important for efficient accumulation of Cdc3G at the growth pole. This is in accord with the altered steady-state distribution of Cdc3G in benomyl-treated hyphae (Fig 2A). Consistently, FRAP analysis of hyphae expressing Rrm4R^{RRMA} also revealed an elevated $t_{1/2}$ of recovery of 12 min (Fig 5C, supplementary Fig S4B).

For comparison we analysed the recovery of constitutively expressed cytoplasmic Gfp. Recovery was in the order of seconds

(supplementary Fig S4C–D) indicating that free cytoplasmic Cdc3G would reach the growth pole within seconds, again arguing for an assembly process [17].

An alternative hypothesis is that endosomal mRNA transport could deposit *cdc3* mRNA at the growth pole for local translation followed by incorporation into septin filaments. However, bleaching the hyphal tip should have excluded that newly translated and matured Gfp fused to Cdc3 contributed to the fluorescence recovery measured at this site. By irradiating the whole filament with moderate laser intensity we photobleached the vast majority of Gfp molecules and measured the recovery by newly translated and matured Gfp. Within 15 min we did not measure an increase in fluorescence (supplementary Fig S4E–F) confirming that *de novo* synthesis of protein takes substantially longer than the measured half live of recovery of 6 or 12 min and thus, does not contribute substantially to the recovery of our FRAP analysis. Therefore it is highly unlikely that mRNA transport and local translation at the hyphal tip constitutes a relevant process (see below).

Septin Cdc12 is essential for endosomal localisation of Cdc3

Septins constitute important cellular proteins involved in cytokinesis and membrane dynamics [13,18]. Their assembly into higher-order structures is essential for septin function. According to current views, septins form heterooligomeric complexes [13]. A possible function for endosome-coupled translation could be the assembly of septin building blocks on endosomes for subsequent delivery. To obtain indications for such a process, we analysed a second septin Cdc12 that directly interacts with Cdc3 in *S. cerevisiae* [18].

Figure 5. Endosomal septin transport is important for its accumulation in filaments.

A Representative FRAP experiment analysing hypha expressing Cdc3G (supplementary Movie S10; asterisk marks septum).

B, C FRAP analysis of Cdc3G at hyphal tips of indicated strains (data were fitted to uniphasic exponential equation, dotted lines indicate half time of recovery; $n = 6$ and $n = 3$ in (B) and (C), respectively; error bars represent s.e.m.). Fluorescence is normalised to plateau, further details in supplementary Fig S4.

Cdc12G also localised in filaments (supplementary Fig S5A) that, however, did not form a gradient emanating from the hyphal tip. This suggests that the stoichiometry of septin building blocks within filaments can differ between subcellular regions of the hyphae. Interestingly, comparable to Cdc3G, Cdc12G also localised to bidirectionally moving units that shuttle throughout the filament (supplementary Fig S5B). Movement was inhibited by benomyl treatment (supplementary Fig S5B) and Cdc12G signals co-localised with FM4-64 (supplementary Fig S5C) demonstrating endosomal shuttling of Cdc12G. Importantly, in hyphae of *cdc12Δ* strains, Cdc3 did not localise to shuttling endosomes (supplementary Fig S5D). Moreover, loss of Cdc12 disturbed the formation of Cdc3G-containing septin filaments and caused defects in unipolar growth (60% bipolar hyphae 6 h p.i.; supplementary Fig S5E). These data are consistent with our hypothesis that at least some steps in septin assembly take place on endosomes and might be a prerequisite for the formation of functional septin filaments.

Conclusions

Studying mRNA transport revealed that *cdc3* mRNA is an *in vivo* target of the Rrm4-dependent transport machinery that hitchhikes on endosomes (supplementary Fig S6A; [3]). Two scenarios are conceivable to explain this observation. In the classic view, the endosomal mRNA transport machinery would deliver the mRNA in concert with the translation apparatus to the growing tip (supplementary Fig S6B). Translation in this region would lead to synthesis of Cdc3 protein, which is incorporated into filaments. Alternatively, loading of endosomes with septin protein might be mediated by endosome-coupled translation of *cdc3* mRNA (supplementary Fig S6C). At present, we cannot rule out with certainty that transport and local translation takes place at the hyphal tip, but based on the following *in vivo* observations we favour the second hypothesis: (i) FRAP experiments excluded that newly synthesised protein generated by translation at the hyphal tip contributes substantially to the observed microtubule-dependent recovery of septin Cdc3 in filaments at the growth cone. (ii) RNA live imaging did not reveal any deposition or accumulation of *cdc3* mRNA at the hyphal tip. (iii) *cdc3* mRNA and the encoded protein co-localise at identical shuttling endosomes. (iv) Cdc3 protein only localises to the Rrm4-positive endosomal compartment if mRNAs are present, while Cdc3 protein that is translated in the cytoplasm is unable to associate with shuttling endosomes. Note that loss of Rrm4 did not result in a decrease of *cdc3* mRNA or protein amount (supplementary Fig. 2F and G). (v) Ribosomal proteins of both subunits co-localise with shuttling endosomes, and this depends on the presence of mRNA. Consistently, also the localisation of Pab1 on endosomes is dependent on mRNA [3]. (vi) Finally, Cdc3 protein is hardly found on shuttling endosomes in the yeast form. This correlates with our previous finding that RNA binding by Rrm4 is substantially lower in yeast cells [8].

Active bi-directional transport of mRNAs without an obvious destination has been widely observed and seems like a waste of energy. Thus, its cellular role has been a long-lasting mystery [19,20]. In mammalian neurons, for example, intensive shuttling of mRNAs is observed without strong accumulation at specific subcellular sites. This process was compared to a sushi belt continuously serving demanding synapses with mRNAs [4]. Here, we provide a

novel explanation for these phenomena: mRNAs shuttle while being locally translated on moving endosomes. This cell biological process might not be restricted to *U. maydis*, because Kinesin-3 type motors like Kif1B, which are known to function in vesicular transport, are also involved in mRNA trafficking in neurons [21,22]. Moreover, septins were previously purified from presynaptic vesicles and they exhibit vesicle-like staining patterns in neurons [13].

In general, endosomes mainly receive their cargo from other membranous compartments by fusion with transport vesicles [23]. We here uncover that septin protein can also be loaded onto moving endosomes by local translation comparable to the aerial refuelling of planes. This new mechanism targets the cargo to the correct subcellular destination opening up additional unanticipated insights into protein trafficking via these multipurpose platforms.

Materials and Methods

Plasmids, strains, and growth conditions

Standard molecular biology techniques were applied and growth conditions were described previously [3,24].

Microscopy and FRAP experiments

Standard microscopy was carried out as described before [3]. RNA live imaging and co-localisation studies were performed using a laser-based epifluorescence microscopy set-up (Zeiss Axio Observer.Z1; [3]). An area of 15 μm from hyphal tips was bleached with 10% laser power (80 mW nominal power VisiFRAP 2D-System, Visitron Systems, Munich, Germany).

Benomyl and FM4-64 staining

Benomyl was added to 20 ml of liquid culture (50 μM f.c.) that was grown for at least one additional hour at 28°C and 200 rpm before microscopical analysis. For FM4-64 staining an aliquot of the filament suspension was labelled with 0.8 μM FM4-64 (Life Technologies, Darmstadt, Germany). After 30–60 s of incubation at room temperature samples were analysed microscopically.

Supplementary information for this article is available online: <http://embor.embopress.org>

Acknowledgements

We acknowledge Dr. K. Zarnack and lab members for discussion and reading of the manuscript. We are grateful to P. Happel, U. Gengenbacher and S. Esch for technical assistance and to Drs. H. Tschochner and M. Leibundgut for insights on the tagging of ribosomal proteins. S. Zander, Drs. T. Pohlmann and R. Kahmann supported this study. The work was funded by grants from the DFG to MF (FE 448/3-2).

Author contributions

MF, SB and JKö designed this study and analysed data. JKö, JKo and SB performed wet bench experiments. SB established the RNA live imaging system, performed microscopic analysis, quantification and data evaluation. MF and SB wrote the manuscript with input from all co-authors. MF directed the project.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Rusten TE, Vaccari T, Stenmark H (2012) Shaping development with ESCRTs. *Nat Cell Biol* 14: 38–45
2. Yap CC, Winckler B (2012) Harnessing the power of the endosome to regulate neural development. *Neuron* 74: 440–451
3. Baumann S, Pohlmann T, Jungbluth M, Brachmann A, Feldbrügge M (2012) Kinesin-3 and dynein mediate microtubule-dependent co-transport of mRNPs and endosomes. *J Cell Sci* 125: 2740–2752
4. Doyle M, Kiebler MA (2011) Mechanisms of dendritic mRNA transport and its role in synaptic tagging. *EMBO J* 30: 3540–3552
5. Pratt CA, Mowry KL (2013) Taking a cellular road-trip: mRNA transport and anchoring. *Curr Opin Cell Biol* 25: 99–106
6. Eliscovich C, Buxbaum AR, Katz ZB, Singer RH (2013) mRNA on the move: the road to its biological destiny. *J Biol Chem* 288: 20361–20368
7. Vollmeister E, Schipper K, Baumann S, Haag C, Pohlmann T, Stock J, Feldbrügge M (2012) Fungal development of the plant pathogen *Ustilago maydis*. *FEMS Microbiol Rev* 36: 59–77
8. Becht P, König J, Feldbrügge M (2006) The RNA-binding protein Rrm4 is essential for polarity in *Ustilago maydis* and shuttles along microtubules. *J Cell Sci* 119: 4964–4973
9. König J, Baumann S, Koepke J, Pohlmann T, Zarnack K, Feldbrügge M (2009) The fungal RNA-binding protein Rrm4 mediates long-distance transport of *ubi1* and *rho3* mRNAs. *EMBO J* 28: 1855–1866
10. Koepke J, Kaffarnik F, Haag C, Zarnack K, Luscombe NM, König J, Ule J, Kellner R, Begerow D, Feldbrügge M (2011) The RNA-binding protein Rrm4 is essential for efficient secretion of endochitinase Cts1. *Mol Cell Proteom* 10: M111.011213 1–15.
11. Alvarez-Tabares I, Perez-Martin J (2010) Septins from the phytopathogenic fungus *Ustilago maydis* are required for proper morphogenesis but dispensable for virulence. *PLoS ONE* 5: e12933
12. Böhmer C, Ripp C, Bölker M (2009) The germinal centre kinase Don3 triggers the dynamic rearrangement of higher-order septin structures during cytokinesis in *Ustilago maydis*. *Mol Microbiol* 74: 1484–1496
13. Spiliotis ET, Gladfelter AS (2012) Spatial guidance of cell asymmetry: septin GTPases show the way. *Traffic* 13: 195–203
14. Göhre V, Vollmeister E, Bölker M, Feldbrügge M (2012) Microtubule-dependent membrane dynamics of *Ustilago maydis*: trafficking and function of Rab5a-positive endosomes. *Comm Integra Biol* 5: 482–487
15. Schuster M, Sreedhar K, Fink G, Collemare J, Roger Y, Steinberg G (2011) Kinesin-3 and dynein cooperate in long-range retrograde endosome motility along a non-uniform microtubule array. *Mol Biol Cell* 22: 3645–3657
16. Dobbelaere J, Gentry MS, Hallberg RL, Barral Y (2003) Phosphorylation-dependent regulation of septin dynamics during the cell cycle. *Dev Cell* 4: 345–357
17. Sprague BL, McNally JG (2005) FRAP analysis of binding: proper and fitting. *Trends Cell Biol* 15: 84–91
18. Barral Y (2010) Cell biology. Septins at the nexus. *Science* 329: 1289–1290
19. Bullock SL (2011) Messengers, motors and mysteries: sorting of eukaryotic mRNAs by cytoskeletal transport. *Biochem Soc Trans* 39: 1161–1165
20. Zimyanin VL, Belaya K, Pecreaux J, Gilchrist MJ, Clark A, Davis I, Johnston D (2008) *In vivo* imaging of *oskar* mRNA transport reveals the mechanism of posterior localization. *Cell* 134: 843–853
21. Charalambous DC, Pasciuto E, Mercaldo V, Pilo Boyl P, Munck S, Bagni C, Santama N (2013) KIF1Bbeta transports dendritically localized mRNPs in neurons and is recruited to synapses in an activity-dependent manner. *Cell Mol Life Sci* 70: 335–356
22. Lyons DA, Naylor SG, Scholze A, Talbot WS (2009) Kif1b is essential for mRNA localization in oligodendrocytes and development of myelinated axons. *Nat Genet* 41: 854–858
23. Huotari J, Helenius A (2011) Endosome maturation. *EMBO J* 30: 3481–3500
24. Brachmann A, König J, Julius C, Feldbrügge M (2004) A reverse genetic approach for generating gene replacement mutants in *Ustilago maydis*. *Mol Gen Genom* 272: 216–226