En bloc TGN recruitment of Aspergillus TRAPPII reveals TRAPP maturation as unlikely to drive RAB1-to-RAB11 transition

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ABSTRACT

Transport protein particle (TRAPP) complexes regulate membrane traffic. TRAPPII and TRAPPIII share a core hetero-heptamer, also denoted TRAPPI. In fungi TRAPPII and TRAPPIII mediate GDP exchange on RAB1 and RAB11, respectively, regulating traffic across the Golgi, with TRAPPII also activating RAB1 in autophagosomes. Our finding that Aspergillus nidulans TRAPPII can be assembled by addition of a TRAPPII-specific subcomplex onto core TRAPP prompted us to investigate the possibility that TRAPPII and/or TRAPPIII already residing in the Golgi matures into TRAPPII to determine a RAB1-to-RAB11 conversion as Golgi cisternae progress from early Golgi to TGN identity. By time-resolved microscopy, we determine that the TRAPPII reporter Trs120 (the homolog of metazoan TRAPPC9) is recruited to existing trans-Golgi network (TGN) cisternae slightly before RAB11 arrives, and resides for ∼45 s on them before cisternae tear off into RAB11 secretory carriers. Notably, the core TRAPP reporter Bet3 (the homolog of metazoan TRAPPC3) was not detectable in early Golgi cisternae, being instead recruited to TGN cisternae simultaneously with Trs120, indicating en bloc recruitment of TRAPPII to the Golgi and arguing strongly against the TRAPP maturation model.

KEY WORDS: GEF, Golgi, RAB GTPase, Exocytosis, Multisubunit tethering complex

INTRODUCTION

Transport protein particle (TRAPP) complexes regulate membrane traffic, acting as guanine nucleotide exchange factors (GEFs) on the RAB GTPases RAB1 and RAB11 (Jones et al., 2000; Morozova et al., 2006; Cai et al., 2008; Pinar et al., 2015; Thomas and Fromme, 2016; Riedel et al., 2017; Thomas et al., 2018, 2019; Pinar et al., 2019). The activation of RAB1 and RAB11 has been shown to be governed by RAB11 (Pantazopoulou et al., 2014). In both A. nidulans and S. cerevisiae, the activation of RAB11 at the TGN is mediated by TRAPPII (Morozova et al., 2006; Pinar et al., 2015, 2019; Riedel et al., 2017; Thomas et al., 2019), thereby reducing the problem of mechanistically understanding the transition between TGN and SV identity to determining how TRAPPII is assembled on, or recruited de novo to, TGN cisternae.

In recent work, we established that certain mutations destabilizing TRAPPII result in the formation of two stable subcomplexes, one consisting of the core hetero-hexamer (i.e. TRAPPI) and the second, denoted the TRAPPII-specific subcomplex, consisting of Trs120, Trs130, Trs65 and Tca17 (Pinar et al., 2019). This raised the possibility that TRAPPII is actually assembled by addition of the whole TRAPPII-specific subcomplex onto TRAPP core hetero-heptamers already residing in the Golgi. This possibility was very attractive, given that changes in the composition of TRAPPs shift their substrate specificity from RAB1, activated by TRAPPII/TRAPPIII, to RAB11, activated by TRAPPIII (Morozova et al., 2006; Pinar et al., 2015, 2019; Riedel et al., 2017; Thomas et al., 2018, 2019). Thus, TRAPP maturation could drive the transition of RAB1 into RAB11, thereby governing traffic across the Golgi by programmed RAB conversion (Morozova et al., 2006; Rivera-Molina and Novick, 2009).

Using fluorescence microscopy, we determine that TRAPPII is present in exocytic membranous compartments in addition to...
localizing to pre-autophagosomal structures (PASs), and study the consecutive arrival of TRAPPII and RAB11 to extant TGN cisternae. Using 3D and 4D microscopy, we determined that Bet3 strictly colocalizes with TRAPPII on TGN cisternae over time, rebuttering a model in which the conversion of TRAPPI/TRAPPIII into TRAPPII governs the maturation of RAB1 into RAB11 and thus traffic across and exit from the Golgi.

RESULTS

Revisiting the localization of the TRAPPIII component Trs85

In fungi, RAB1 plays roles in autophagy (Lynch-Day et al., 2010; Pinar et al., 2013b), in addition to exocytic roles in the ER/Golgi interface (Lord et al., 2011) and the TGN (McDonold and Fromme, 2014). It was widely accepted that TRAPPIII would only regulate the autophagic functions of RAB1, whereas TRAPPI would regulate the Golgi functions. However, a recent report concluded that S. cerevisiae TRAPPI does not exist as such, and that it is actually TRAPPIII that regulates all functions of RAB1, including those in the ER/Golgi interface and in the TGN (Thomas et al., 2018). This conclusion was buttressed by recent data strongly indicating that TRAPPI is present in very minor amounts, if at all, in A. nidulans (Pinar et al., 2019). However, the role of TRAPPIII required further investigation because partial localization of Trs85 to Sec7-containing Golgi cisternae in non-autophagic yeast cells (Thomas et al., 2018) is consistent with the TGN role but inconsistent with the ER/Golgi role.

A. nidulans growth is exquisitely sensitive to secretory pathway impairment. trs85Δ causes a colony growth defect (Fig. 1C) that is difficult to reconcile with an strictly autophagic role of TRAPPIII [atgΔ mutations do not affect growth (Pinar et al., 2013b)]. Thus, we revisited the localization of Trs85, comparing it with that of every other TRAPP component, endogenously tagged with fluorescent proteins. With the exception of Trs33 and Trs65, all these other proteins are essential. Therefore, that the corresponding strains are

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viable indicates that tagging does not preclude function. Trs85–GFP is also functional, as judged by its ability to restore the growth defect resulting from trs85A (Fig. S1). Trs120–GFP localizes to TGN cisternae (Pinar et al., 2015) (see below). Fig. 1D shows that all GFP-tagged core TRAPP and TRAPPII reporters localize similarly to Trs120–GFP, that is they localize to TGN-like cisternae, strongly polarized towards the tip, with faint staining of the SPK. [For Bet5, we used Bet5–mCherry (mCh), as Bet5–GFP was largely cytosolic.] Using similar image acquisition conditions, Trs85–GFP clearly accumulated on pre-autophagosomes (PASs), circumscribed to regions located far away from the tips (Fig. 1E) (Pinar et al., 2013b). In addition, Trs85–GFP was weakly localized to the SPK, as well as to a cytosolic ‘haze’ against which numerous faint small puncta were barely noticeable and ‘empty’ nuclei appeared as ‘fluorescence-free’ ovals (Fig. 1E, arrowheads). Increased exposure times combined with image deconvolution served to confirm the presence of Trs85–GFP fluorescence at the SPK and made the presence of the small puncta, clearly predominating ahead of the apicalmost nucleus, more apparent against the cytosolic haze (Fig. 1F). The definition of these puncta was further improved after endogenously tagging Trs85 with tandemly triplicated GFP (GFPx3) (Fig. 1F) (the growth test shown in Fig. S1 shows that Trs85–GFPx3 is also functional). These TRAPPIII (Trs85) puncta are clearly distinct from TGN cisternae in that they are markedly smaller and far more numerous than the latter (compare cells in Fig. 1D and E, F displayed at the same magnification). That Trs85–mCh localized like Trs85–GFP (Fig. 1E) argues against the possibility that the observed Trs85 localization was a tagging artifact.

The faint fluorescence of the small Trs85 puncta, as well as the strong cytosolic haze, precluded in vivo colocalization studies. However, the presence of Trs85 at the SPK (an accumulation of secretory vesicles) points to a role for TRAPPII in the exocytic pathway, and it is tempting to speculate that the faint, polarized punctate structures containing Trs85, that are different in size and abundance from TGN cisternae, reflect the activation of RAB1 by TRAPPII at early Golgi compartments (see also Discussion).

### The TRAPPII component Trs120 cycles on TGN cisternae

A previous study with *S. cerevisiae* concluded that Trs31 (a core TRAPP) colocalized with TRAPPII, but not with an early Golgi marker, suggesting that TRAPP complex(es) other than the TGN-located TRAPPII were difficult to visualize by fluorescence microscopy (Montpetit and Conibear, 2009). In contrast, another study concluded that Bet3 (also a core TRAPP), although colocalizing with TRAPPII, did so incompletely (Thomas et al., 2018). Because Golgi maturation processes might affect the localization of TRAPP complexes over time, for example, if TRAPPI/TRAPPIII itself ‘matures’ into TRAPPII, we decided to investigate the colocalization of a core TRAPP subunit with the TRAPPII-specific Trs120 reporter by time-resolved microscopy, aiming to detect a class of cisternae containing the former but not the latter. To improve the time resolution (*Tr*) of previous studies (Pinar et al., 2015), we tagged Trs120 endogenously with GFPx3. Trs120–GFPx3 is markedly brighter than Trs120–GFP, supports vigorous growth and requires lower exposure time, improving *Tr* and allowing for longer periods of imaging without cell photodamaging. Trs120–GFPx3 localizes to polarized TGN cisternae (Fig. 2A), whose average lifetime is 2 min (Pantazopoulou et al., 2014). Trs120 is recruited at these cisternae only after they are formed and stays until the end of their cycle, when they dissipate into post-Golgi carriers (Pinar et al., 2015).

Consequently, cisternae visualized with the ‘structural’ TGN marker Sec7–tdTomato (tdT) are more abundant than those detected with Trs120–GFPx3 (a proportion of Sec7–tdT cisternae has not yet acquired Trs120) (Fig. 2B), which results in partial colocalization (Fig. 2C) (Pearson’s coefficient 0.61±0.05, mean±s.d., *n*=19 cells).

The kymograph shown in Fig. 2D, derived from Movie 1 consisting of 210 middle planes of a hypha acquired with a *Tr* of 2 fps, illustrates how Trs120–GFPx3 is recruited to TGN cisternae and resides on them until they ’tear off’ into post-Golgi carriers bound for the apex. These cycles of recruitment/departure are reflected by slightly tilted, vertical lines whose lengths correspond to the lifetime of Trs120 (TRAPPII) on cisternae. The also tilted line drawn by the growing apex shows that the hypha continues growing (at 0.9 µm min⁻¹) during imaging, demonstrating that these are bona fide *in vivo* observations. Similar kymographs of 3D time series were obtained with Trs120–tdTomato (Fig. S2), Tca17–GFPx3 and Trs130–GFP (Fig. S3A, B, left), showing that fluorescent reporters reflect the behavior of TRAPPII.

To discard the possibility that the recurrence of Trs120–GFPx3 captured by kymographs represented cisternae going in-and-out of focus, we acquired 4D (*x, y, z, t*) movies, such that each frame was the MIP projection of a Z-stack (Movie 2 shows a hypha growing at 0.63 µm s⁻¹). Kymographs captured a pattern closely resembling that seen with middle planes (Fig. 2F), which was similarly seen using another TRAPPII-specific reporter, Trs130–GFP (Fig. S3B, right) (with 4D time-series a smaller *Tr* results in shorter vertical lines). Thus, the recurrence pattern of fluorescent reporters does not result from cisternae moving out of focus and rather reflects the transient residence of TRAPPII at TGN puncta. Reassured by these results, we analyzed *n*=78 maturing TGN cisternae extracted from movies acquired with a *Tr* of 1 fps from eight different Trs120–GFPx3 cells, and determined that the time of residence of TRAPPII in TGN cisternae was 44±9 s (mean±s.d.) (Fig. 2E).

Trs120 precedes RAB11 in arrival to TGN cisternae

The above TRAPPII time-of-residence is approximately one-third of the ~2 min lifetime of a TGN cisterna (Pantazopoulou et al., 2014). As Trs120 (TRAPPII) mediates nucleotide exchange on RAB11, the latter should be recruited to cisternae in parallel to, or after Trs120 accumulates. RAB11 and Trs120 roughly coincide (at low *Tr* of 0.33 fps) in their arrival to the TGN, at the end of the cisternal cycle, before cisternae acquire post-Golgi identity and depart towards the SPK (Pinar et al., 2015). To increase *Tr* we developed a bright mCherry–RAB11 reporter that we combined with Trs120–GFPx3 to acquire 4D movies, recording the red and green channels simultaneously with a beam splitter every 1.2 s (i.e. with a *Tr* of 0.83 fps). Notwithstanding that RAB11 strongly accumulates in the SPK, the two markers colocalized substantially on TGN cisternae (Fig. 3A; Movie 3). Indeed kymographs (Fig. 3B) clearly illustrated the similar patterns of arrival and departure of Trs120 and RAB11, and confirmed that Trs120 substantially colocalizes with RAB11 over time. The strong apical RAB11 signal hindered systematic analysis, but visual examination of the life cycles of *n*=43 TGN cisternae showed that in 70% the arrival of Trs120 slightly preceded that of RAB11 (Fig. 3B shows an example). That Trs120 slightly precedes RAB11 was confirmed by exploiting the higher *Tr* (2.2 fps) of 3D movies made with middle planes (Fig. 3C).

Core TRAPP subunits also cycle on Golgi cisternae

For co-imaging with Trs120–GFPx3, we chose Bet3 as it contributes two subunits to the complex, which showed to be the...
brightest among our panel of fluorescent protein-tagged core proteins. Bet3 localized to Golgi-like puncta irrespective of whether it was tagged with GFP, tdT or mCherry, indicating that this represents its physiological localization (Fig. 4A; Fig. S4). Bet3–tdT, consistently most resistant to photodamage, was chosen for colocalization studies. 4D microscopy revealed transient Bet3–tdT localization to cisternae (Movie 4), as seen with Trs120 and Trs130. Fig. 4B shows a kymograph of a Bet3–tdT 2 min sequence with a Tr of 1 fps. As with Trs120, the Bet3-containing SPK is seen as a continuous, tilted line reflecting apical extension growth, whereas the transient recruitment of Bet3 to cisternae is reflected in characteristic slightly tilted, vertical lines. This pattern in which the pool of a TRAPP core component builds up in Golgi cisternae before eventually dissipating was confirmed with Trs23–GFPx3 (Fig. S3C).

**Fig. 2. Transient recruitment of TRAPPII to the TGN.** (A) Localization of Trs120–GFPx3 to polarized TGN cisternae. The positions of nuclei (n) are indicated in the linescan. A.U., arbitrary units. (B) Colocalization of Trs120–GFPx3 with Sec7, endogenously tagged with Sec7–tdTomato using simultaneously acquired channels. Images are maximum intensity projections of deconvolved Z-stacks. (C) Pearson’s coefficient analysis of Trs120 and Sec7 colocalization (n=19 cells). (D) Kymograph derived from a Trs120–GFPx3 3D movie acquired with indicated parameters. (E) Time of residence of Trs120–GFPx3 on 78 maturing TGN cisternae obtained from n=8 cells. (F) Kymograph derived from a Trs120–GFPx3 4D movie acquired with indicated parameters. Error bars on graphs represent mean±s.d.

**Bet3 and Trs120 colocalize continuously, disproving the TRAPP conversion model**

Next we tried to detect a Golgi compartment containing the TRAPP core but not TRAPPII. In the prevailing model, TRAPP/TRAPPIII activates RAB1 in the early Golgi whereas TRAPPII activates RAB11 in the TGN (Morozova et al., 2006; Thomas et al., 2019). According to this model, Bet3, which is required for activating both RAB1 and RAB11, should be distributed between early Golgi and TGN cisternae, which are optically resolvable (Pantazopoulou and Peñalva, 2011; Arst et al., 2014; Hernández-González et al., 2014).
Fig. 3. TRAPPII slightly precedes RAB11 in their arrival to TGN cisternae. (A) Colocalization of mCh–RAB11 and Trs120–GFPx3. Note the strong signal of RAB11 accumulating at the SPK. The two channels were acquired simultaneously. Images are maximum intensity projections of deconvolved Z-stacks. (B) Inverted grayscale kymographs for mCh–RAB11 and Trs120–GFPx3, derived from a 4D movie in which the two channels were simultaneously acquired with beam splitter (indicated by DW), using the indicated parameters. The insets including the merge of the two channels show an example of Trs120 accumulating at a TGN cisterna before RAB11 does. Arrowheads indicate the arrival of RAB11 (magenta) and Trs120 (green) to the cisterna. Note that mCh–RAB11 strongly accumulates in the SPK. (C) Example of Trs120–GFPx3 clearly preceding mCh–RAB11 at the cisterna. The kymograph was derived from a 3D movie of middle planes acquired at 2.2 fps with a beam splitter and a 2×2 binning.

Fig. 4C shows that Bet3–tdT and Trs120–GFPx3 completely colocalized (Pearson’s coefficient 0.897±0.045, mean±s.d.; Fig. 4D), strongly indicating that a vast majority of core TRAPP is present in cisternae that contain TRAPPII (i.e. TGN cisternae). This result was confirmed after examining visually 667 Bet3–tdT Golgi cisternae (38 hyphae, two independent experiments); 666 contained Trs120, indicating that subclasses of cisternae containing TRAPPI/TRAPPIII only or TRAPPII only do not exist or are below detection by wide-field epifluorescence microscopy. Thus, Bet3 solely localizes to Trs120-containing TGN cisternae. Indeed Bet3–tdT did not colocalize with early Golgi cisternae marked with the GFP-tagged syntaxin Sed5 (Fig. 4C) (Pearson’s coefficient 0.138±0.06, mean±s.d., Fig. 4D), whereas it did colocalize with Sec7–GFP to an extent similar to Trs120–tdT (Fig. 4C) (Pearson’s of 0.448±0.02 and 0.532±0.06, mean±s.d., respectively; Fig. 4D). (As noted above, incomplete colocalization of Bet3 and Trs120 with the Sec7 TGN marker is attributable to the narrow window-of-residence of TRAPP subunits on TGN cisternae.) Taken together, these experiments argue against distinct TRAPP complexes acting at the transition of early Golgi to TGN cisternae. However, they do not completely rule out the possibility that Bet3 and Trs120 coincide on the same cisternae only transiently, during the period of time in which a hypothetical ‘maturation’ of TRAPPI/TRAPPIII into TRAPPII would take place in parallel to cisternal maturation. This might occur, for example, if TRAPPII were assembled onto a pre-existing Golgi-localized core TRAPP by addition of TRAPPII-specific subunits (Morozova et al., 2006). To address this possibility, we acquired time-series of Bet3–tdT and Trs120–GFPx3. Fig. 5, Fig. S5A,B and Movie 5 show that the two reporters completely colocalized over time in 3D sequences having a TR of 1–1.4 fps. Moreover, 4D series at 1 fps demonstrated that the coincident pattern of temporal residence of Bet3 and Trs120 on Golgi cisternae does not result from fluorescent puncta going in-and-out of focus (Movie 6; Fig. S5C). Thus, either fluorescent Bet3 forms part of different TRAPPI/TRAPPIII and TRAPPII complexes locating to the same TGN cisternae, or Bet3 and Trs120 belong to the same (TRAPPII) complexes. That Bet3 and Trs120 signals colocalize completely and coincide in their relative intensities, shapes and short-range movements strongly supports the latter interpretation, refuting the TRAPP conversion model.

TRAPPII is the predominant TRAPP complex in Aspergillus, being twice as abundant as TRAPPIII (Pinar et al., 2019). In budding yeast a substantial amount of TRAPPIII colocalizes with Sec7 and TRAPPII (Thomas et al., 2018). Thus, we explored the possibility that the high levels of TRAPPII present in TGN puncta mask the presence of core TRAPP subunits associated with TRAPPII co-existing on the same cisterna. To do this, we deleted trs120Δ in a strain carrying a constitutively active RAB11 allele (denoted RAB11*, encoding RAB11ΔD125E) to restore viability (Pinar et al., 2019), which results in the complete absence of TRAPPII (Pinar et al., 2019). Bet3–tdT* became almost completely dispersed in trs120Δ RAB11* cells, with few faint, small puncta that were hardly noticeable over the resulting cytosolic haze (Fig. 6A). As RAB11* by itself does not affect the TGN localization of Bet3–tdT (Fig. S6), this delocalization is attributable to trs120Δ. The observation that Bet3–tdT-containing TGN cisternae were not at all noticeable in trs120Δ cells is important because it strongly suggests that TRAPPII does not mask the hypothetical presence of substantial amounts of TRAPPI (or TRAPPII) on TGN membranes. Thus, to reinforce this conclusion we performed the same experiment with another core TRAPP reporter, Trs23–GFPx3, which gave essentially the same results (Fig. 6B). Unfortunately, the identity or dynamics of the few faint, small cytosolic puncta containing TRAPPIII core proteins could not be addressed by colocalization studies and time-lapse microscopy, as these structures were almost completely obscured by the cytosolic haze.
This is visually illustrated by the kymographs in Fig. 6C, which compare the sharp pattern of recurrence of TRAPP on TGN membranes in the wild-type with the hazy pattern of Trs23–GFPx3 in a trs120Δ RAB11* cell (vertical ‘empty’ sections correspond to nuclei), with hardly any structure noticeable on the hazy background. Of note, as trs120Δ markedly increases the levels of TRAPPI at the expense of TRAPPII, but does not affect those of TRAPPIII (Pinar et al., 2019), the cytosolic pattern of core TRAPP markers resulting from TRAPPII ablation would be attributable to the artificial increase in TRAPPI, suggesting that the core TRAPP is not targeted to the Golgi.

We next tested whether TRAPPIII localization was altered by ablating TRAPPII after performing similar experiments with Trs85–GFPx3 in wild-type and trs120Δ RAB11* cells (Fig. 6D). The localization of Trs85/TRAPPIII to a cytosolic haze, the SPK and small punctate structures was essentially undisturbed by trs120Δ (Fig. 6D), showing that TRAPPIII localization is TRAPPII-independent.

**DISCUSSION**

We exploited here the suitability of *A. nidulans* to study intracellular traffic by *in vivo* fluorescence microscopy. In this fungus, intracellular transport involving cooperation between actin- and microtubule-based motors (Abenza et al., 2009, 2010; Zhang et al., 2011, 2014; Taheri-Talesh et al., 2012; Peñalva et al., 2017) resembles that of metazoan cells to a greater extent than that in *S. cerevisiae*. *A. nidulans* hyphal tip cells are particularly useful to investigate the secretory pathway because impairment of exocytosis results in morphological defects (Pinar et al., 2013a,b; Hernández-González et al., 2014), which can facilitate, for example, assessment of whether endogenous tagging of TRAPP subunits with fluorescent proteins negatively affects their function. The Golgi, consisting of scattered early and TGN cisternae, is polarized towards the growing tip (Pantazopoulou and Peñalva, 2009, 2011; Pantazopoulou et al., 2014; Hernández-González et al., 2019), reflecting that exocytosis is targeted to the apex to maintain the supply of cell wall-modifying enzymes and lipids required for rapid growth by apical extension (~1 μm/min at 28°C on microscopy wells) (Hernández-González et al., 2018a). The organization of the Golgi facilitates the analysis, which can be undertaken using kymographs such as those extensively used here, of the fate of large numbers of cisternae over time, which has been exploited to document the maturation of TGN cisternae into post-Golgi RAB11 compartments.
TGN cisternae are transient structures that, at the end of their 2 min lifecycle, become RAB11 carriers destined for the plasma membrane (Pantazopoulou et al., 2014). TRAPPII, which arrives slightly before RAB11, is not an integral component of TGN cisternae, accumulating on them only when a certain stage of maturation is reached. This observation explains why Trs120 colocalizes only partially with Sec7, a constituent marker of the TGN (a proportion of Sec7 cisternae are captured at a maturation stage in which they might not have yet accumulated detectable levels of TRAPPII). The accumulation of TRAPPIII in the TGN brings about the activation/recruitment of RAB11, which marks the Golgi-to-SVs transition and the engagement of molecular motors for the transport of these SVs to the SPK (Pehalva et al., 2017; Hernández-González et al., 2018a) (Fig. 7).

An important finding of this work was that A. nidulans Trs85, the diagnostic subunit of TRAPPIII, is not exclusive to the PASs, being also detectable in other structures, including SVs concentrated at the SPK, revealing an unexpected and unexplored link between TRAPPIII and exocytosis. Of note, BapH, an effector of RAB11 and RAB1, is not precluded by these observations, permitting the activation of RAB1 for its exocytic role. In previous work, we classified TRAPP subunits according to their essential roles in activating RAB1, RAB11 or both using ‘constitutive’ mutations of the GTPases that bypass the requirement for their GEFs (Pinar et al., 2019). For example, the viability of trs120Δ (TRAPPIII) mutants could be rescued by expression of constitutively active RAB11 alone, whereas that of a bet3Δ (core) mutant necessitated expression of both constitutively active RAB1 and RAB11, in accordance with the role of certain core hetero-heptamer subunits in the activation of both GTPases (Pinar et al., 2019).

TRAPPII does not exist in S. cerevisiae (Thomas et al., 2018) and if it exists at all in A. nidulans it would be in minor quantities (Pinar et al., 2019), difficult to detect by microscopy, such that its hypothetical presence in low abundance in the Golgi might be obscured by highly abundant TRAPPII. However, we show here that in trs120Δ cells lacking TRAPPII, in which a major proportion of TRAPP is artificially shifted to TRAPPII, the core components Bet3 and Trs23 are delocalized to a cytosolic haze. This hazy pattern indicates that TRAPPI is unable to localize to Golgi cisternae, contributing to mounting evidence that it is TRAPPIII rather than TRAPPI that mediates RAB1 activation in the Golgi.

We show that a vast majority of A. nidulans TRAPP localizing to the Golgi is accounted for by TRAPPII present at the TGN. Previous work uncovered the possibility that TRAPPII is assembled by adding the stable TRAPPII-specific subcomplex containing Trs120, Trs130, Trs65 and Tca17 onto core TRAPP hetero-heptamers (Pinar et al., 2019). This modular TRAPPII assembly would provide mechanistic support for the model by which core TRAPP already localized to the Golgi would mature into TRAPPI as cisternal maturation progresses, determining the transition between RAB1 and RAB11 stages (Morozova et al., 2006). As this core TRAPP is unlikely to proceed from TRAPPII, it would be TRAPPIII which would mature into TRAPPII by substituting TRAPPIII-specific subunits with TRAPPI-specific ones. In this scenario, Trs85 in TRAPPIII would compete with Trs120 in TRAPPI for its Trs20 component Trs120 strictly overlaps the TGN cisterna cannot be visualized with core TRAPP reporters (Fig. 6), even though trs120Δ does not affect the amount of both constitutively active RAB1 and RAB11, in accordance with the role of certain core hetero-heptamer subunits in the activation of both GTPases (Pinar et al., 2019).
of TRAPPIII present (Pinar et al., 2019). Finally, trs120Δ does not affect the normal localization of Trs85-labeled TRAPPIII to a cytosolic haze and to weakly bright puncta that are clearly smaller than TGN cisternae, suggesting that TRAPPI and TRAPPIII do not compete for the same core TRAPP. This apparent absence of TRAPPIII in the Aspergillus TGN contrasts with the situation in S. cerevisiae, where a detectable amount of TRAPPIII colocalizes with the TGN reporter Sec7 (Thomas et al., 2018). We note, however, that we cannot rule out the possibility that small, faint Trs85 puncta represent specialized domains of TGN cisternae. Addressing this possibility will require developing brighter fluorescent reporters for Trs85 combined with super-resolution microscopy.

In any case, the maturation model predicts that a proportion of Golgi cisternae should contain TRAPP core subunits but not Trs120. However, still images of core TRAPP and TRAPPII-specific components showed that all localized to TGN-like puncta. Moreover, the Bet3 core TRAPP component showed a similar degree of colocalization with Sec7 to Trs120, arguing against the above possibility. As Trs23 and Bet3 ‘cycle’ on TGN cisternae in a similar manner to what is seen with Trs120, we then considered the possibility that core components peak on TGN cisternae before TRAPPI-specific components do. However, Bet3–tdT and Trs120–GFPx3 completely colocalized over time, both appearing and dissipating simultaneously, which argues against them accumulating at different time points of the cisternal cycle. Instead, the complete colocalization, the coincident shapes of Bet3 and Trs120 kymograph traces, and the correlating brightness of the two reporters strongly indicate that Bet3 and Trs120 belong to the same TRAPPII complexes, negating the ‘TRAPP maturation’ model.

A key implication of these findings is that TRAPPII is recruited as a whole to extant TGN cisternae. The TGN mediates endocytic recycling both in yeast (Day et al., 2018) and in A. nidulans (Hernández-González et al., 2018a), but efficient endocytic recycling in the latter fuels hyphal growth, which might have resulted in species-specific features in the regulation of the TGN. It is plausible that TRAPPII monitors, and responds to, the arrival at TGN cisternae of membranous carriers of endocytic origin that

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**Fig. 6.** Localization of core TRAPP components and the TRAPPIII component Trs85 in the absence of TRAPPII. (A) Localization of Bet3–tdT in the wild-type and in trs120Δ cells lacking TRAPPII (rescued with RAB11*). Images are maximum intensity projections (MIPs) of Z-stacks that were not deconvolved to preserve the original signal intensities. (B) Localization of Trs23–GFPx3 in the wild-type and in trs120Δ cells lacking TRAPPII (rescued with RAB11*); Z-stack-derived images were acquired as in A. (C) Kymographs depicting the localization of Trs23–GFPx3 over time, derived from movies acquired with a T of 1 fps. (D) Trs85-GFPx3 (TRAPPIII) localization is not affected by ablation of TRAPPII. Images are MIPs of deconvolved Z-stacks. All cells are shown at the same magnification.
MATERIALS AND METHODS

Aspergillus media, culture conditions and transgenes

Standard A. nidulans media were used for strain propagation and conidiospore production (Pinar and Peñalva, 2017; Hernández-González et al., 2018b). Genetic techniques were used as described previously (Todd et al., 2017). Strains are listed in Table S1.

Endogenous gene tagging with fluorescent protein tags

A plasmid containing the rab11 (also known as rabE) promoter upstream of the mCherry–RAB11 coding region, followed by the 3' UTR of the gene and the A. fumigatus pyrG gene, used as selective marker, was integrated in the rab11 locus as described for its GFP–RAB11 equivalent (Pantazopoulou et al., 2014), such that the resulting strains contained a untagged copy of rab11 besides the mCherry-tagged one. The correct integration event was confirmed by Southern blotting. All other genes were endogenously tagged with fluorescent proteins using PCR-assembled linear DNA cassettes and homology-driven recombination. Single GFP and the mCherry coding regions, were fused in frame to the GFPx3 coding region, thus generating an intermediate plasmid into which PCR DNA fragments, flanked by NotI and XmaI sites at their 5' and 3'-ends, respectively, and containing gene-specific 3'-flanking regions fused to the A. fumigatus pyrG gene, were cloned in the intermediate plasmid after NotI and XmaI digestion. The complete cassette was excised after digestion with SbfI and XmaI and used to select pyrimidine independent transformants of MAD5736 or MAD6512, as appropriate, to generate tagged strains carrying compatible pairs of auxotrophic markers for subsequent crossing.

Microscopy

Hyphae imaged by epifluorescence microscopy were cultured in pH 6.8 'watch minimal medium' (WMM) (Petalva, 2005) at 28°C, using eight-well chambers (IBIDI GmbH, Martinsried, Germany or Lab-Tek, Nalge Nunc International, Rochester, NY). Images were acquired with Leica DMi6000 B inverted microscopes equipped with Leica 63×/1.4 N.A. Plan Apochromatic objectives and driven by Metamorph Premier software (Molecular Dynamics). The temperature of the cultures was controlled with a Heating Insert P on-stage incubator (Leica) combined with an objective heater (PeCon GmbH, Germany), as described previously (Pinar et al., 2013a), or with a Leica incubation hood enclosing the whole microscope.

Leica EL6000 light sources provided excitation light. The microscopes were coupled to Hamamatsu ORCA-ER CCD (1344×1024 pixels, 6.45 µm cell size) and Orca-Flash 4.0LT CMOS (2048×2048 pixels; 6.5 µm cell size) cameras. Single-channel acquisition of fluorescence images was made with Semrock GFP-3035B and TXRED-4040B 'BrightLine' filter cubes. All colocalization experiments were made with images acquired simultaneously in the two channels with either a Photometrics Dual-View beam splitter (CCD camera) or a Gemini beam splitter (Hamamatsu) equipped with Chroma ET632/60 and Semrock 01-512/25 emission filters (CMOS camera). The dual excitation filter in the microscope was a Chroma 95022-ET for EGFP/mCherry. Precise alignment of Dual View channels was carried out using fluorescent beads (TetraSpeck microspheres, blue/green/orange/dark red; Molecular Probes) as references and the 'color-align' menu of Metamorph software. Alignment of the Gemini channels used the software provided by the manufacturer.
3D (x, y, t) and 4D (x, y, z, t) stacks of images were acquired with the minimal settings of the excitation source that provided a sufficient signal, with an appropriate exposure time, to prevent cell photodamage. To improve time resolution in 4D acquisitions, Z-stacks of images acquired with a Z-pass of 0.25–0.5 µm were transferred to the computer RAM using the ‘stream acquisition’ function of Metamorph. Channels were merged using the ‘color align’ Metamorph plug in. Movies of maximal intensity projections of the Z-stacks were constructed with the ‘review multidimensional acquisition’ plugin of Metamorph. Annotated movies were converted to QuickTime format using ImageJ and file size-adjusted using mpeg-4 compression.

Image manipulation and analysis

All still images shown correspond to Z-stacks deconvolved with Huygens Professional for Win64. Images and time series were converted to 8-bit monochrome or 24-bit RGB and annotated with Corel Draw (Corel, Ottawa, Canada). For colocalization analyses using the Pearson’s coefficient, Z-stacks of images in the GFP and red (tdT or mCherry) channels acquired with a beam splitter were deconvolved with Huygens software. Pearson’s coefficients were determined with the Coloc 2 plugin of FIJI (ImageJ 1.52), using the corresponding maximal intensity projections and manually traced regions of interest (ROIs) covering the apex-proximal region of the hyphae containing the greatest abundance of Golgi cisternae and excluding the nuclei. Datasets for each combination of markers were analyzed with Prism 3.02 (GraphPad software). To determine the effects of trs33A in the recruitment of Trs120–GFP to TGN cisternae, 5 µm-deep Z-stacks of wt and trs33A cells expressing Trs120–GFP were acquired with the same settings. Maximal intensity projections of these images were thresholded to include only the fluorescence signal present in Golgi puncta in the apical-most 20 µm of the hyphae and the total amount of signal within the threshold area was calculated and plotted with Prism 3.02.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.P., M.A.P.; Methodology: M.P., M.A.P.; Validation: M.P., M.A.P.; Formal analysis: M.P., M.A.P.; Investigation: M.P., M.A.P.; Resources: M.P.; Writing – original draft: M.P.; Writing – review & editing: M.P., M.A.P.; Visualization: M.A.P.; Supervision: M.A.P.; Funding acquisition: M.A.P.

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