The Golgi apparatus: insights from filamentous fungi

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Abstract: Cargo passage through the Golgi, albeit an undoubtedly essential cellular function, is a mechanistically unresolved and much debated process. Although the main molecular players are conserved, diversification of the Golgi among different eukaryotic lineages is providing us with tools to resolve standing controversies. During the past decade the Golgi apparatus of model filamentous fungi, mainly *Aspergillus nidulans*, has been intensively studied. Here an overview of the most important findings in the field is provided. Golgi architecture and dynamics, as well as the novel cell biology tools that were developed in filamentous fungi in these studies, are addressed. An emphasis is placed on the central role the Golgi has as a crossroads in the endocytic and secretory-traffic pathways in hyphae. Finally the major advances that the *A. nidulans* Golgi biology has yielded so far regarding our understanding of key Golgi regulators, such as the Rab GTPases RabC^{Rab6} and RabE^{Rab11}, the oligomeric transport protein particle, TRAPP^{II}, and the Golgi guanine nucleotide exchange factors of Arf1, GeaA^{GBF1/Gea1} and HypB^{BIG/Sec7}, are highlighted.

Key words: *Aspergillus nidulans*, filamentous fungi, Golgi apparatus, intracellular traffic, secretion

THE GOLGI APPARATUS IN EUKARYOTES: LABORS, ATTRIBUTES AND DEBATES

Secretion is essential for organisms, both during development and for maintaining life. In rapidly growing filamentous fungi, for example, secretion maintains cell-wall expansion (Riquelme 2013); in the presynaptic neuronal axis of multicellular organisms it mediates release of neurotransmitters for body coordination (Sudhof 2013); in social insects secretion of trail pheromones mediates coordination of the colony (Noirot and Quennedey 1974, Billen and Morgan 1998, Meer and Preston 2008). Cells also synthesize continuously and sort proteins and lipids destined to replenish protein and membrane that are lost due to cell functions (e.g. endocytosis, multivesicular body sorting) or to change the composition and function of locales in response to ambient changes; for example, to provide

the plasma membrane with transporters specific for the detected nutrients (Pantazopoulou and Dhalluin 2007); in other cases macromolecules are being secreted to modify the cell environment or for self-protection.

In eukaryotes the central hub of the (conventional) secretory pathway is the Golgi apparatus, functionally situated between the endoplasmic reticulum (ER) and the final destinations of secretory cargo (Mironov and Pavelka 2008b). The Golgi receives biosynthetic cargo from the ER. While at the Golgi proteins are post-translationally modified, mostly glycosylated, and membranes change their lipid composition (Mironov and Pavelka 2008a). Meanwhile the Golgi also receives recycling cargo from the endo-lysosomal system and it thus is considered a crossroads of exocytosis and endocytosis (Mironov and Pavelka 2008a). Eventually cargo is sorted into carriers to be delivered to the endosomal system, the plasma membrane or to the extracellular milieu. Overall post-translational protein modifications, remodeling of the lipid bilayer and cargo sorting appear to be the major Golgi labors in the cell (Munro 2011b).

One feature of the Golgi apparatus is intra-organelle compartmentalization. Discrete Golgi units are called cisternae. The roots of the word trace back to the Latin “cista”, from ancient Greek “κίστη” (kiste), meaning box or basket, from Proto-Indo-European kista, meaning woven container (American Heritage Dictionary of the English Language, 4th ed., “cisterna,” <http://www.yourdictionary.com/cistern> [accessed 23 Oct 2015], Oxford Dictionaries, “cist,” <http://oxforddictionaries.com/definition/english/cist> [accessed 23 Oct 2015] and Wiktionary via the Liddell-Scott-Jones Greek-English lexicon, 8th ed.), which justifies using it to refer to individual Golgi units of any morphology, in agreement with the terminology used by (Bourett et al. 2007) for the fungal Golgi and not just to the flattened saccules present normally in higher eukaryotes. Cisternae making up the Golgi apparatus can be classified according to various interdependent characteristics, such as their content in enzymes that glycosylate biosynthetic cargo (Farquhar 1985, Graham and Emr 1991) or in proteins that regulate membrane identity (Munro 2005), their lipid composition that defines the physical properties of the bilayer (Holthuis and Menon, 2014), as well as the membrane-traffic pathways operating to and from them (Papanikou and Glick 2014). Post-translational modification-mediating enzymes and lipids form gradients through different classes of cisternae, from where cargo is considered to pass in an ordered way, such that the Golgi appears as a polarized organelle. There is an uncertainty as to

Submitted 5 Nov 2015; accepted for publication 1 Jan 2016.

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FIG. 1. Filamentous-fungal Golgi in electron micrographs. A. Golgi cisternae seen as tubules or sheets belong to the same organelle, as suggested from electron micrographs of adjacent, serial, thin sections of freeze-substituted cells of *Fusarium acuminatum*. Some cisternae are hollow spheres. The arrows indicate tubular elements that exhibit continuity with Golgi sheets and tubules but are wider and more electron dense. Bar = 500 nm. Images are reproduced from Howard RJ. (1981). Ultrastructural analysis of hyphal tip cell growth in fungi: Spitzenkörper, cytoskeleton and endomembranes after freeze-substitution. *J Cell Sci* 48:89–103, PMID: 7196918, with permission of the Journal of Cell Science. B. Golgi cisternae (GB) in transmission-electron micrographs of near median sections through *Aspergillus nidulans* germ tube apical regions. Arrowheads: Woronin bodies. Asterisks: Spitzenkörper vesicles. M = Mitochondria. Bar = 250 nm. Image reprinted from Momany M, Richardson EA, van Sickle C, Jedd G. (2002). Mapping Woronin body position in *Aspergillus nidulans*. *Mycologia* 94:260–266. C. The Golgi is organized in stacks (GB) in *Monoblepharis macrandra* cells. Li = lipid bodies, M = mitochondria, MVB = multivesicular bodies. Bar = 500 nm. Image reprinted from Dee JM, Mollicone M, Longcore JE, Roberson RW, Berbee ML. (2015). Cytology and molecular phylogenetics of Monoblepharidomycetes provide evidence for multiple independent origins of the hyphal habit in the Fungi. *Mycologia* 107:710–728.

how many fundamentally different Golgi compartments (i.e. groups of same-class cisternae) there are in cells. Opinions range from a minimum of two, the Trans Golgi network (TGN) from where cargo exits the Golgi and the earlier cisternae that accept ER traffic (Rothman, 1981), to three, four or more compartments (Brigance et al. 2000, Papanikou and Glick, 2014).

The Golgi is a highly dynamic organelle: many of its resident proteins cycle continuously through the ER or the endosomes, while upon treatment with Brefeldin A (BFA) (a drug inhibiting the activation of the central Golgi regulator Arf1 [Robineau et al. 2000]), the mammalian *cis*-/medial-Golgi merges with the ER and the TGN mixes with recycling endosomes only to reappear after BFA removal (Lippincott-Schwartz et al. 1989, Lippincott-Schwartz et al. 1991). During mitosis

the mammalian Golgi undergoes fragmentation only to reorganize once mitosis is complete (Colanzi and Corda 2007).

How are diverse cisternae maintained (albeit in a dynamic equilibrium), while a continuous flow of cargo finds its way through the Golgi? This question has generated much debate for more than 50 y now and has yielded various models intending to explain cargo passage through the Golgi. The cisterna maturation model prevails, postulating that cisternae form de novo, change their identity over time, by acquiring next stage-determining molecules and by recycling back the earlier stage molecules and eventually dissipate in secretory carriers, while cargo progresses along with the maturing cisternae toward the locale for which it is destined. However, it has gradually become obvious that this model is insufficient to explain all

experimental observations, leading to integration of modifications to its central theme (some excellent reviews on this debate can be found in Glick and Luini [2011] and Mironov et al. [2013]). A condensed introduction to the field of Golgi biology can be found in Munro [2011b], while a detailed analysis of the status of Golgi research, up to some years ago, can be found in Mironov and Pavelka [2008b]).

FILAMENTOUS FUNGAL GOLGI IN ELECTRON MICROGRAPHS

Freeze-substitution cell fixation in electron microscopy permitted the observation of “Golgi equivalents” in various filamentous fungal species that once were believed to lack this organelle (Howard, 1981). In electron micrographs, both in Ascomycota (*Fusarium acuminatum* [Howard, 1981]) and Basidiomycota (*Laetisaria arvalis* [Hoch and Howard, 1980]), individual cisternae are dispersed in the cytoplasm, instead of forming the well-packed stacks present in animals and plants (Polishchuk and Mironov 2004). In serial thin sections, “Golgi-like elements”, that sometimes were found in the proximity of mitochondria, appeared to consist of interconnected smooth-surfaced fenestrated sheets of various orientations, or fenestrated hollow spheres, as well as tubules (Howard 1981). An excellent example of such a series of sections, revealing the detailed structure of *F. acuminatum* cisternae, is illustrated (FIG. 1A). In *Aspergillus nidulans* hyphae (Momany et al. 2002, Bourett et al. 2007) (FIG. 1B), as well as in the conidiophore stalk, metulae and phialidae (Mims et al. 1988), Golgi bodies also are dispersed and their distribution is polarized toward the tip of the stalk and sterigmata. Studies in other Dikarya (such as *Coprinopsis cinerea* [McLaughlin 1974], *Armillaria melea* [Grove and Bracker 1970], *Aspergillus niger* and *Verticillium albo-atrum* [Grove and Bracker 1970] and *Neurospora crassa* [Girbardt 1969]), although realized before the introduction of freeze-substitution for fixation of hyphae, also had suggested that elements that are not organized in stacks possibly are Golgi bodies. However, using the more distant fungal group of Monoblepharomycetes, it was shown that hyphal growth is not exclusively linked to dispersed Golgi, as hyphae of *Monoblepharis macrandra* were shown to possess Golgi stacks (Dee et al. 2015) (FIG. 1C). The Golgi apparatus among yeasts has similar diversification in its organization (Glick 1996): dispersed cisternae are observed in *Saccharomyces cerevisiae* (Preuss et al. 1992), while stacks of cisternae are present in *Schizosaccharomyces pombe* (Chappell et al. 1994) and *Pichia pastoris* (Gould et al. 1992). It has been proposed that existence of either one or the other Golgi organization reflects the underlying differences in the organization of the endoplasmic reticulum (ER) exit sites: In *P. pastoris*, where the

Golgi forms stacks, ER exit sites are discrete, confined at specific ER locales, while in *S. cerevisiae*, where the Golgi is dispersed, vesicles bud throughout the ER network (Rossanese et al. 1999). Golgins, large coiled-coil proteins at Golgi’s cytoplasmic surface, together with Golgi ReAssembly Stacking Proteins (GRASPs) also have been implicated in Golgi stacking, as well as in Golgi ribbon formation in mammalian cells (Wang et al. 2003, Munro 2011a, Lee et al. 2014, Veenendaal et al. 2014), while these proteins are thought to contribute to the specificity of membrane traffic to the Golgi in yeast but also in higher eukaryotes (Behnia et al. 2007, Wong and Munro 2014). Nevertheless, the exact mechanism by which stacking is achieved is not definitively understood and even less clear remains the role this Golgi organization plays in protein secretion or other cellular function (Emr et al. 2009).

IS STUDYING THE GOLGI COMPLEX IN FILAMENTOUS FUNGI WORTHY?

The origin of the Golgi complex can be traced back to the origin of the eukaryotic cell. Most of the molecular machineries that operate in the organelle specifying its membrane identity and permitting directionality in membrane flow (e.g. the SNARES, Arf and Rab family small GTPases and their regulators, COP vesicle coats, the adaptor protein complexes and subunits of tethering complexes such as the transport protein particle [TRAPP] I/II and the conserved oligomeric Golgi complex [COG]) already existed in the last common eukaryotic ancestor (Jékely 2008). That being said, it is the Golgi diversification observed among eukaryotic lineages that has been instrumental in diagnosing the elements essential for Golgi’s basic labors and that has provided us with specialized tools for the study of conserved Golgi functions. For example, the fact that Golgi is dispersed in *S. cerevisiae* and thus individual cisternae can be chased by optical microscopy has been exploited to test in vivo whether cisternae are stable compartments or if they form de novo, change their identity over time and finally dissipate in secretory carriers (i.e. if they mature) (Losev et al. 2006, Matsuura-Tokita et al. 2006). This would have been impossible in mammalian cells because the distances of cisternae within a stack fall below optical resolution, which impedes pursuit of individual cisternae through time. However, yeast is not a cure-all: For example, unlike in mammalian cells or in long filamentous fungi where intracellular traffic is highly demanding (Schmitz and Philippsen 2011, Zhang et al. 2011, Egan et al. 2012, Penalva et al. 2012, Schuster et al. 2012, Riquelme 2013, Pantazopoulou et al. 2014, Take-shita et al. 2014), microtubules are not used in yeast for either the traffic of endosomes or of secretory carriers,

suggesting that perhaps yeast Golgi exit and thus organization of the TGN somehow would be specialized. It is gradually becoming clear that more model systems need to be put into play to dissect an organelle as complex and as ancient as the Golgi. In addition, studies of the Golgi in filamentous fungi are permitting us to knowingly look for and interfere with secretion bottlenecks in these organisms that long have been used as cell factories without an in-depth understanding of their secretory pathways (Archer 2000, Conesa et al. 2001, Riquelme 2013, Meyer et al. 2015).

THE GOLGI ARCHITECTURE UNDER THE FLUORESCENCE MICROSCOPE

It took almost 25 y from electron micrographs (and the development of live cell microscopy techniques) before light was shed again on the filamentous fungal Golgi. In one of the first efforts, heterologous expression of yeast Ypt1p (Rab1 homolog; a master Golgi regulator) fused to the green fluorescent protein (GFP) in *Ustilago maydis* showed labeling mostly of the hyphal apex and septa but also faintly of scattered cytoplasmic bodies (Wedlich-Soldner et al. 2002). In one of the early studies with endogenous proteins, CopA, the *A. nidulans* alpha subunit of the heptameric CopI coat mediating intra-Golgi and Golgi-to-ER retrograde transport (Popoff et al. 2011), was used as a reporter fused to GFP. CopA::GFP was seen to decorate hollow “doughnut-like” structures scattered in the cytoplasm (Breakspear et al. 2007), consistent with cisternae morphology in electron micrographs and resembling the 1,2-alpha-mannosidase-labeled structures that had been observed in *Aspergillus oryzae* (Akao et al. 2006). CopA::GFP was shown to colocalize with the red fluorescent protein (RFP)-tagged transmembrane domain of alpha-2, 6-sialyltransferase (Hubbard and Kaminskyj 2008), a Golgi enzyme. The *A. nidulans* GPD-mannose transporters GmtA and GmtB co-localized in Golgi-like puncta (Jackson-Hayes et al. 2008, 2010). Cytosolic puncta negative for CMAC staining also were observed with some of the GFP-labeled *A. oryzae* Golgi-related SNARE homologs, such as AoGos1p (Kuratsu et al. 2007). The unique *A. nidulans* homolog of yeast Arf1p/Arf2p, ArfA (small GTPases of the Arf family are master Golgi regulators), localized to Golgi-like bodies similar to those visualized by CopA::GFP, and this localization was dependent on the ArfA myristoylation site, Gly2, as ArfA^{G2A}::GFP mutant was cytoplasmic (Lee and Shaw 2008) in agreement with the notion that myristoylation ensures tight membrane association of the Arf family (Donaldson and Jackson 2011). Upon BFA treatment that prevents Golgi guanine nucleotide exchange factors (GEFs) from activating Arf1 (Arf1 activation is thought to be necessary for its Golgi membrane

localization [Gillingham and Munro 2007]), ArfA was shown to relocate to larger and less numerous structures than the Golgi-like bodies. The authors suggested that this phenotype was consistent with ArfA Golgi-like bodies being BFA-sensitive, strengthening the idea that they represent Golgi cisternae (Lee and Shaw 2008). Although studies with CopA and ArfA had been instructive, in both cases expression of the *gfp*-tagged alleles was driven by the *alcA* promoter from extra gene copies, such that a potential localization artifact, caused by non-physiological expression or a possible alteration in protein function due to the GFP tag, could not have been ruled out.

Our group used a dual strategy to label the Golgi and assess its dynamics (Pantazopoulou and Penalva 2009): On the one hand we GFP-tagged the ArfA-GEF Sec7, an essential Golgi regulator (Kaminskyj and Hamer 1998, Arst et al. 2014), by in locus gene replacement, maintaining its native promoter. The strain carrying the *sec7::gfp* allele had no apparent growth defect, which indicated that the transgene, expressed at naturally relevant levels, was functional, and thus would offer a bona fide Golgi image. On the other hand, we constructed a robust reporter, highly fluorescent and photo-stable enough to follow Golgi dynamics over time. We did this by tagging with mRFP the pleckstrin homology domain of the oxysterol binding protein (PH^{OSBP}), which had been shown to localize at the Golgi due to coincidence detection of Arf1 and phosphatidylinositol-4-phosphate (PI4P) (Levine and Munro 2002). mRFP::PH^{OSBP}-labeled structures resembled the Sec7::GFP-labeled ones. Indeed mRFP::PH^{OSBP} co-localized with Sec7::GFP in growing hyphae, which suggested that mRFP::PH^{OSBP} is also a reliable Golgi reporter. Detailed study of this reporter revealed that the Golgi apparatus in *A. nidulans* has these characteristics: (i) it consists of ring-shaped and fenestrated cisternae with tubular extensions, resembling in their form the cisternae seen in electron micrographs; (ii) PH^{OSBP}-labeled cisternae show a largely polarized distribution, with the bulk of fluorescence concentrating toward the tip, ahead of the leading nucleus (but with an exclusion zone [in the range of a few micrometers] immediately below the apex; FIG. 2A); (iii) cisternae are generally dispersed throughout the cytoplasm, but it is clear, both by the faint tubules seen to connect them (FIG. 2A) and by their short-range movements that sometimes appear concerted, that cisternae are part of a network, rather than isolated; (iv) this network is highly dynamic in at least two ways: First, cisternae undergo continuous morphological transformations, changing shapes, while fluorescence intensity fluctuates and seems to redistribute between adjacent structures, and, second, cisternae have short-range non-directional movements and

sometimes external forces would appear to pull or stretch them (Pantazopoulou and Penalva 2009). Expression of the artificial mRFP::PH^{OSBP} reporter did not have any effect on hyphal growth, and furthermore, mRFP::PH^{OSBP} seemed to monitor Golgi activity, as, in response to anaerobiosis and/or mechanical pressure resulting from mounting hyphae on slides, it re-localized rapidly to the cytoplasm (mRFP::PH^{OSBP}, similar to Sec7::GFP, is a peripheral membrane protein, expected to become cytoplasmic when its localization requirements on membranes are not met). This made clear that, so as to be meaningful, studies of an organelle as dynamic as the Golgi should be carried out in undisturbed growing hyphae (for example, in liquid medium observed by an inverted microscope in chambers kept at the appropriate temperature and in illumination conditions minimizing photodamage). It soon became obvious that Golgi architecture as observed by fluorescence microscopy correlated with growth speed, in that perturbations of Golgi integrity were readily reflected in apical extension (Pantazopoulou and Penalva 2009, 2011). Moreover, alterations in Golgi organization, achieved, for example, by modifying the ER-to-Golgi interface flux using conditional mutants (*sarA^{ts}*, *rabO^{ts}*, *sedV^{ts}*, see also below), resulted in tip morphology alterations. This revealed a potential way to achieve hyphal depolarization, which is necessary for shifts in the morphogenesis plan during, for example, appressorium formation in *Magnaporthe oryzae* or conidiophore development in *A. nidulans* (Harris 2013, Pinar et al. 2013, Hernandez-Gonzalez et al. 2015).

DECIPHERING GOLGI COMPARTMENTALIZATION IN HYPHAE

A functional Golgi unit in higher eukaryotes consists of diverse cisternae that are organized in a stack. Cisternae have varying biochemical characteristics that correlate with their relative position in the stack. A typical mammalian interphase cell includes 40–100 stacks connected by membrane tubules to form a perinuclear Golgi ribbon, while in invertebrates stacks do not form a ribbon but instead are dispersed in the cytoplasm (Wei and Seemann 2010). In *Drosophila melanogaster* dendrites, *cis*-, medial- and *trans*-Golgi cisternae appear to be disconnected (not forming stacks) in vivo (Zhou et al. 2014). In microsporidia the Golgi is a tubular network connected with the ER and the plasma membrane that comes in two morphologies (plane or varicose tubules [Beznoussenko et al. 2007]). How does filamentous fungal Golgi (that consists of cisternae that in most cases are [i] scattered in the cytoplasm, [ii] not organized in stacks, [iii] frequently interconnected by tubules and [iv] polarized toward

the growing apex) relate to these examples with regard to its compartmentalization and the organization of the different compartments that build up the cell's Golgi apparatus? One important question was whether absence of stacks in hyphae is due to dispersion of cisternae with distinct identity or if it reflects a potential lack of functional compartmentalization in fungal Golgi. In other words: Do dispersed cisternae in fungi have the biochemical differences observed in organisms with stacks?

The early and late Golgi in A. nidulans.—In *S. cerevisiae* fluorescent-tagged protein markers characteristic of the late (corresponding to *trans* in cells with stacks) or the early (corresponding to *cis*) Golgi are present in distinct sets of cisternae scattered over the cytoplasm that are seen as puncta resolvable by optical microscopy (Wooding and Pelham 1998).

In *A. nidulans* a compartment labeled with the Sed5 syntaxin homolog, SedV, segregates from the PH^{OSBP}-labeled compartment (Pantazopoulou and Penalva 2011). Further localization studies with other Golgi markers revealed that indeed we can distinguish two main Golgi territories. For example, GFP::RerA, homolog of Rer1, a membrane protein receptor responsible for the retrieval of ER resident proteins from the Golgi (Sato et al. 1997), showed extensive co-localization with mCherry::SedV and clear segregation from mRFP::PH^{OSBP} (Pinar et al. 2013) (FIG. 2A). GeaA::GFP, a member of the family of early Golgi Arf1-GEFs (GBF1/Gea), also co-localized substantially with mCherry::SedV, while segregating from mRFP::PH^{OSBP} (Arst et al. 2014). GFP::TlgB (TlgB is the Tlg2/syntaxin16 homolog) co-localized with mRFP::PH^{OSBP} at the late territory, while mRFP::TlgB segregated from GFP::SedV (Pinar et al. 2013). Sec7::GFP, member of the family of late Golgi Arf1-GEFs (BIG/Sec7), co-localized with mRFP::PH^{OSBP} (Pantazopoulou and Penalva 2009). GrhA, the homolog of GRASP65/Grh1, involved in early Golgi assembly by COPII vesicles (Behnia et al. 2007), segregates from PH^{OSBP}, in addition, GrhA-labeled structures collapse in large aggregates in the presence of BFA (similar to the collapse seen with ArfA [Lee and Shaw 2008]), suggesting that GrhA localizes at the Golgi (collapse into large aggregates is a characteristic response of the Golgi but not of the ER to BFA treatment; see also below and [Pantazopoulou and Penalva 2009]). *A. oryzae* GrhA homolog substantially co-localized with AoVip36 and AoEmp47 (Hoang et al. 2015), two cargo receptors with L-type lectin domains involved in ER to Golgi trafficking of N-linked glycoproteins. Thus SedV, RerA and GeaA substantially co-localize at one class of cisternae (where GrhA might also localize), while

FIG. 2. Filamentous-fungal Golgi compartmentalization. A. Wide-field fluorescence micrographs of early and late Golgi compartments in the *Aspergillus nidulans* tip cell (in the region ahead of the leading nucleus). The late Golgi is labeled by the mRFP::PH^{OSBP} reporter, the early Golgi is labeled by GFP::RerA (see text for details). Note how GFP::RerA-containing cisternae almost reach the apex, while mRFP::PH^{OSBP}-containing cisternae appear to be excluded from the subapical region. The late Golgi compartment is clearly a network of interconnected cisternae, in that faint tubules can be seen to connect the globular, more intensely fluorescent, cisternae. Note that early and late Golgi cisternae intermingle but they are clearly segregated entities.

Tlg2, PH^{OSBP} and Sec7 co-localize at a different class of cisternae (FIG. 2B). The markers we used were functionally suggestive: The only two *A. nidulans* Golgi syntaxins SedV and Tlg2 (López-Berges et al. 2016) mark two distinct compartments by the conserved Arf1-GEFs GeaA and Sec7, members of the cis/early and trans/late subfamily of Golgi Arf1-GEFs, respectively, (Arst et al. 2014) co-localize with the relevant early or late syntaxin; the RerA receptor responsible for the retrieval of ER resident proteins early in the secretory pathway (Sato et al. 1997) is confined with the early Golgi syntaxin and the early Golgi Arf1-GEF, as expected;

the coincidence of PI4P with activated Arf1, both of which have a pivotal role in Golgi exit (D'Souza-Schorey and Chavrier 2006, Daboussi et al. 2012, De Matteis et al. 2013), is detected by the PH^{OSBP} reporter only where the late Golgi syntaxin and the late Arf1-GEF reside. It is important to note that for each of the compartments both transmembrane (SedV, RerA, Tlg2) and peripheral membrane proteins (GeaA, Sec7, PH^{OSBP}) were tagged and localized, minimizing the possibility of artifacts related to the membrane-association mechanism of the hybrid proteins. Accordingly we named the SedV/GeaA/RerA compartment

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Yellow arrow indicates a faintly labeled medial cisterna, where mRFP::PH^{OSBP} and GFP::RerA colocalize. Images are sum projections of deconvolved z-stacks. Metamorph (MolecularDevices) and Huygens Professional Deconvolution (SVI) image analysis software was used. B. Upper panel. A scheme depicting Golgi compartmentalization in filamentous fungi at a time point $\times 1$. Two main compartments are observed: in the early compartment (in green) proteins that are predicted to act early (SedV, RerA, GeaA) in the secretory pathway colocalize. In the late compartment (in red) proteins that are predicted to act late (TlgB, PH^{OSBP}, Sec7) in the secretory pathway colocalize. Protein markers from different compartments mainly segregate (see A). Early and late Golgi markers colocalize in a minority of cisternae, the medial (yellow) cisternae. A fraction of the late Golgi cisternae are labeled by Trs120 and RabE^{Rab11} (purple), and we have shown with time lapses that these cisternae gradually lose Golgi identity, acquiring post-Golgi identity, until they dissipate into secretory carriers that are transported to the apex, presumably to maintain polarized growth. Lower panel. Depiction of the lifecycle of an individual cisterna over time (cisterna "M" of the upper panel), based on data and hypotheses. The x-axis represents time. Cisternae are transient (Pantazopoulou et al. 2014, unpubl). Late Golgi cisternae mature to RabE^{Rab11/Ypt31}-containing post-Golgi carriers (Pantazopoulou et al. 2014, Pinar et al. 2015) and early Golgi cisternae mature to medial and then to late Golgi (Pantazopoulou and Peñalva unpubl). Golgi Rabs partially localize at the early and/or the late Golgi. Because Rabs are determinants of identity, this indicates that the attributes of cisternae, relating to the traffic pathways to and from them, cannot be accurately described by their simple classification as early, medial and late. The scheme depicts a highly hypothetical, alternative classification of cisternae into the Golgi assembly, carbohydrate synthesis and carrier formation stages based on Day et al. (2013). The early Golgi should exchange traffic with the ER (1) (see text for details) and thus be, at least for some part of its lifetime, at the Golgi assembly stage. After its assembly an early Golgi cisterna would participate in intra-Golgi traffic pathways that would result in its enrichment in glycosylation enzymes by the delivery of early Golgi resident proteins (EGR) originating from other early (2) and/or medial (3) Golgi cisternae (carbohydrate synthesis stage). The lipid remodeling that most possibly takes place throughout might at a point trigger the recruitment by the early Golgi of peripheral membrane late Golgi-determinant proteins (LGR) from the cytoplasm (such as Sec7) (4a) resulting in the transformation of early to medial (and then late) Golgi. Hypothetically late Golgi transmembrane proteins (LGR), such as TlgB, might be supplied biosynthetically and distributed by rapid partitioning to late Golgi lipid bilayers through (hypothetical) connections between heterologous Golgi cisternae or they might be delivered by retrograde traffic from the late Golgi to the early/medial Golgi (4b). We know that retrograde pathways most probably operate from the endosomes (ENDO) and/or the post-Golgi endosome (PGE) toward both the early and the late Golgi (5), with the Golgi syntaxins SedV or TlgB being implicated in each step (López-Berges et al. 2016). These routes might even contribute to maturation: In a hypothetical scenario late Golgi resident proteins escaping the Golgi along with cargo bound to the endo-lysosomal system would have the chance to be retrieved from the endosomes and to be recycled back to maturing (early/medial) cisternae (5), contributing to cisternae transformation. Lipid segregation or COPI-dependent retrograde traffic should segregate early from late compartments in the medial cisternae (3). Intra-Golgi traffic based on COPI and/or RabC might take place up to the initial phases of the late Golgi (6). At some point, however, cisternae would be committed to carrier formation. We have observed the HypA^{Trs120}-dependent maturation of cisternae to RabE^{Rab11}-containing carriers bound to the apical plasma membrane (7) (Pantazopoulou et al. 2014, Pinar et al. 2015), but previous or concurrent HypB^{Sec7}-dependent routes most possibly also would regulate the transport of cargo bound to the endosomes (8). Arrows are colored according to the origin (for entry routes) or the destination (for exit routes) compartment: blue for the ER, brown for endosomes, red for the late Golgi, green for the early Golgi and mint green for secretory carriers toward the SPZ. Routes involving the medial Golgi are colored according to their individual early or late components. x- and y-axis shall be used to interpret arrows: Because of their directionality and slope arrows contain information indicating whether routes connect later with earlier (and vice versa) or equivalent (for vertical to the x-axis arrows) compartments.

“early” (*cis*) and the Tlg2/Sec7/PH^{OSBP} “late” (*trans*), depending on the order in which the relevant Golgi markers are predicted to act in the secretory pathway (FIG. 2B).

The medial Golgi.—Segregation of the early from the late Golgi markers, however, is not complete. mRFP::PH^{OSBP} co-localizes with GFP::SedV to some extent (20% of mRFP::PH^{OSBP} bodies have detectable GFP::SedV [Pantazopoulou and Penalva 2009]), which is consistent with the transient co-localization observed between the early and late Golgi markers during cisternal maturation in yeast (Losev et al. 2006, Matsuura-Tokita et al. 2006). Although a putative intermediate in cisternal maturation, this medial compartment may as well classify as a third basic entity of the fungal Golgi: It is distinct in composition from the early or the late compartment and, due to its different composition in molecular traffic determinants, entry/exit pathways to/from the medial cisternae would be different from those in earlier or later cisternae. It might also be that, on the medial compartment, factors typically viewed as early or late Golgi residents are brought close such that interactions between them have an opportunity to occur. For the time being it would be informative to determine whether markers other than SedV::GFP and mRFP::PH^{OSBP} localize at the medial Golgi.

Is classification of the Golgi compartments as early or late sufficient to describe them functionally? The example of localization of the Golgi Rabs.—Deciphering compartmentalization becomes more complex as localization of some Golgi components may come in continuous gradients instead of steep thresholds. In some cases gradients of different markers neither overlap completely nor segregate completely. For example, although Rab proteins are well-established determinants of membrane identity that regulate membrane trafficking (Barr 2013, Casanova et al. 2013), RabC, the *A. nidulans* homolog of Ypt6/Rab6 regulating endosome-to-Golgi, intra-Golgi and Golgi-to-ER retrograde traffic (Liu and Storrie 2012) partially localizes at both the early and late Golgi entities (Pantazopoulou and Penalva, 2011). A similar localization also has been reported for yeast Ypt6 (Suda et al. 2013). Moreover, it seemed that RabC localization is influenced by the distance from the hyphal tip: Close to the tip, where most late Golgi cisternae are found and immediately below the apex, where exocytosis predominates, GFP-RabC localizes mostly in a tubular network containing mCherry-SedV, while away from the tip RabC is in discrete cytoplasmic bodies containing PH^{OSBP} (Pantazopoulou and Penalva 2011). Similarly localization of RabO, the Ypt1/Rab1 homolog regulating

fusion of ER-derived vesicles to form the Golgi (Short et al. 2005), depends on the distance from the apex: At the tip RabO mostly co-localized with SedV at the early Golgi, whereas away from the tip RabO partially co-localized with both SedV at the early and PH^{OSBP} at the late Golgi (Pinar et al. 2013). These data suggested that the Golgi apparatus in long hyphae is not uniform. On the contrary, cisternae location within the cell relative to the growing apex influences their organization. The dependence of Golgi organization on its position in the hypha may reflect possible quantitative differences in Golgi activity depending on the proximity to the apex. Golgi activity in turn may depend on biosynthetic activity (Some data indeed indicate that there are differences in gene expression depending on the nuclear position in hyphae [Kohli et al. 2008, Herrero-Garcia et al. 2015]).

Co-localization quantification methods.—To assess such complex localizations we have used several methods: First, we have been counting how many cisternae show co-localizing signals comparing to the total cisternae number. To do this we determine whether the shapes of cisternae in the two channels appear similar through the x-, y- and z-axis in z-stacks of images to avoid overestimating co-localization due to mere overlaps resulting from the projection of a 3D hypha in two dimensions. In this way we collect binary data on the existence/absence of any degree of apparent co-localization, but we cannot distinguish between potentially very different cases, such as robust co-localization from co-localization of very low with very high signals (very low or high relative to the intensity distribution in each channel). Thus we also have been making use of more sophisticated co-localization analysis tools, especially JACoP plugin (Bolte and Cordelières 2006) of ImageJ (Schneider et al. 2012) and Li’s intensity correlation analysis plots (Li et al. 2004) therein (for an example of the use of the plots, see Pinar et al. [2013]). Still intermediate situations, where markers partially colocalize, are hard to assess accurately in a quantitative manner. In part to circumvent that problem we have been assessing co-localization of markers upon BFA treatment, which triggers Golgi collapse in large aggregates (Lee and Shaw 2008, Pantazopoulou and Penalva 2009). Of note, SedV and PH^{OSBP} localize at distinct BFA aggregates (Pantazopoulou and Penalva 2011), suggesting that these aggregates somehow maintain distinct identities. This method revealed that, for example, RabC localizes partially at both late and early Golgi aggregates while RabO predominates at early Golgi aggregates, although localization at the late Golgi of both of these Rabs in the absence of BFA seemed similar, increasing in tip-distal regions (Pantazopoulou and Penalva 2011, Pinar et al. 2013).

Although we do not currently understand the mechanism of Golgi aggregation upon BFA treatment, it seems logical to assume that differences in BFA reflect some of the existing differences in non-treated cells and thus BFA has been used to assist us in further classifying our ambiguous markers. Improvement in quantification methods, super-resolution microscopy, as well as time-resolved studies of more markers may reveal subdomains in the main Golgi compartments we have defined so far. Co-visualization of more than two markers each time also might help understand the complex localization profiles of proteins that partially localize at both early and late Golgi.

Little is known on Golgi compartmentalization from other filamentous fungi. In *Neurospora crassa*, the homolog of yeast Golgi-localized PMR1 Ca^{++} -ATPase localizes at cytosolic puncta that do not co-localize with the Golgi-associated retrograde protein (GARP) complex subunit VPS-52, suggesting that there are disconnected Golgi subcompartments in hyphae (Bowman et al. 2009). Recently heterokaryons were used for co-localization studies of Golgi determinants and it was found that YPT-1 partially co-localizes with presumed early (USO-1, VRG-4) and late (SEC-7, VPS-52) Golgi markers (Sanchez-Leon et al. 2015). Late Golgi marker SEC-7 and the GARP complex subunit VPS-52 also were found to partially colocalize.

Overall these data show that there is Golgi compartmentalization in filamentous fungi. In *A. nidulans* there are clearly at least two separate entities where essential determinants of the Golgi normally co-cluster: the early and late Golgi compartments that maintain their distinct identities even in the large Golgi-aggregates triggered by BFA. A third compartment, where at least some of the early and late Golgi markers overlap, reflects the continuity over time of the early and late cisternae (i.e. that early cisternae mature to late cisternae). Finally the thresholds delimiting fundamentally distinct functional entities may not completely coincide with the compartment limits set by the markers that, when studied in pairs, determine the early from the late Golgi territories: For example, Golgi Rab proteins seem to transcend the borders of early/late Golgi, suggesting further subcompartmentalization in *A. nidulans*.

GOLGI FUNCTIONAL ENTITIES: THE GOLGI AS CENTRAL HUB OF INTRACELLULAR TRAFFIC PATHWAYS

Because functionality of the Golgi compartments is ultimately conferred by the concurrent localization of numerous proteins and lipids, the localization of a handful of markers cannot be sufficient to understand the functional significance of compartmentalization. To integrate data from different organisms, Day,

Staehelin and Glick recently proposed that three fundamentally different classes of compartments can be distinguished based on the pathways operating to and from them (Day et al. 2013, Papanikou and Glick 2014): (i) the cisternal assembly stage, during which cisternae accept COPII biosynthetic traffic from the ER and deliver back to it COPI-coated vesicles, recycling trafficking components and ER resident proteins, (ii) the carbohydrate synthesis stage, where intra-Golgi transport takes place via COPI-dependent traffic exchange and (iii) the carrier formation stage, during which cisternae receive endosome traffic and form clathrin-coated carriers bound to the endosomes and secretory carriers bound to the plasma membrane (Paczkowski et al. 2015).

The three compartments, early, medial and late, we observe in the *A. nidulans* Golgi most possibly do not reflect these functionally distinct entities. Yet some functional characteristics of the cisternae deduced from the data can be orientating (consult also FIG. 2B).

First, the early Golgi compartment at least for a great part of its lifetime must be at the Golgi assembly stage, as at least two of its residents, RerA and SedV, re-localize to the ER after prolonged BFA treatment or when ER exit is blocked by SarA^{Sar1} conditional mutants (Hernandez-Gonzalez et al. 2015), suggesting that this compartment directly exchanges material with the ER.

Second, the late compartment at least in part must be at the carrier formation stage, in that we have shown that it eventually dissipates into secretory carriers. *A. nidulans* RabE^{RAB11} is the sole homolog of yeast Ypt31 and Ypt32 that regulate polarized secretion (Ortiz et al. 2002, Lipatova et al. 2008). We found that RabE labels post-Golgi carriers (for details see below and Pantazopoulou et al. [2014]). During the last 25 s of its 2-min lifetime the PH^{OSBP} late compartment loses PH^{OSBP} (and Sec7) signal, while being enriched in RabE^{RAB11}. This process concludes with the formation of post-Golgi RabE carriers of long-range motility that are targeted to the Spitzenkörper (SPZ) (Pantazopoulou et al. 2014). We therefore deduce that, when PH^{OSBP} and Sec7 co-localize with RabE, the late Golgi must be at the carrier formation stage, gradually losing Golgi identity while acquiring post-Golgi identity. Based on recruitment of the clathrin-coated-vesicle-specific adaptor GGA on the TGN simultaneously with the peak of Sec7 in yeast (Daboussi et al. 2012, McDonold and Fromme 2014), it is possible that this endosome-bound traffic leaves the TGN before RabE carriers (RabE signal peaks later than Sec7 in *A. nidulans*), thus the duration of the carrier-formation stage might be longer than the period during which RabE stays at the TGN. Shift of the conditional *hypB5^{sec7ts}* mutant (Sec7 regulates at the TGN the formation of clathrin- and GGA-coated vesicles bound to

endosomes) to the restrictive temperature results in Vps10, the sorting receptor of vacuolar proteases that normally cycles between the Golgi and the endosomes, accumulating at the late Golgi (Pantazopoulou and Penalva 2011). This is consistent with Sec7 playing a role in the generation from the late Golgi of Vps10-containing carriers bound to the endosomal system. Because cargo sorting appears to take place while at the carrier formation stage, we can conclude that the late Golgi in *A. nidulans* resembles the TGN of other cells (De Matteis and Luini 2008, Guo et al. 2014).

Functionally discriminating between cisternae based on whether they receive retrograde traffic from endosomes has proven to be complicated in *A. nidulans*. The TlgB-labeled late Golgi compartment most possibly receives endosomal traffic: Tlg2, yeast's homolog of the *A. nidulans* TlgB, and its mammalian homolog, syntaxin 16, both have been implicated in the formation of SNARE bundles regulating fusion with the TGN of vesicles derived from the endosomes (Abeliovich et al. 1998, Amessou et al. 2007). Association of TlgB with the SNARES Vti1, TlgA and SynA observed with *A. nidulans* proteins (López-Berges et al. 2016) is consistent with such a role. *A. nidulans* *rabCΔ* largely re-localizes Vps10 from the Golgi to a cytoplasmic haze, which suggests that RabC regulates an endosome-to-Golgi retrograde pathway. *tlgB* is dispensable but demonstrates synthetic lethality with *rabCΔ*, suggesting a RabC-independent role of TlgB in retrograde traffic (Pantazopoulou and Penalva 2011). Although TlgB must be implicated in retrograde traffic from the endosomes to the Golgi, *tlgBΔ* has a minor effect on the localization of the exocytic R-SNARE SynA (the Snc1p/Snc2p homolog) at an apical crescent (López-Berges et al. 2016) despite the fact that this localization depends on coordination of SynA continuous endocytosis at the subapical endocytic ring with its subsequent redelivery to the apical plasma membrane (Lewis et al. 2000, Valdez-Taubas and Pelham 2003, Taheri-Talesh et al. 2008, Pantazopoulou and Penalva 2011) likely *via* the Golgi (Pinar et al. 2013, López-Berges et al. 2016) as in yeast (Lewis et al. 2000). *tlgAΔ* on the other hand somehow disturbs SynA localization at the apex and reduces its overall presence at the tip, suggesting that it affects SynA recycling more than *tlgBΔ*. Consistently TlgA can interact both with SedV (the early Golgi syntaxin) and TlgB (the late Golgi syntaxin) (López-Berges et al. 2016), suggesting that TlgA also may participate in a TlgB-independent pathway to the Golgi. Moreover, SedV, apart from having been involved in a retrograde pathway through TlgA-dependent SNARE bundles, has been found further to form TlgA-independent bundles with endosomal snares (Syn8-dependent), suggesting

that it perhaps also participates in a retrieval pathway to the Golgi from a later endosomal compartment (López-Berges et al. 2016). This would explain why SynA substantially localizes at the apex even in *tlgAΔ* and is consistent with the double *tlgAΔ tlgBΔ* mutant growing like the wild type. Consistently a mutant debilitating SedV demonstrates synthetic lethality with *tlgBΔ*, further suggesting independent roles of SedV and TlgB in retrograde traffic from the endosomes to the Golgi. In summary, retrograde pathways from the endosomes to the Golgi have been proposed to use at least three distinct SNARE bundles in *A. nidulans*, one involving the late Golgi syntaxin and two involving the early Golgi syntaxin (López-Berges et al. 2016), suggesting that cisternae at both the Golgi assembly and at the carrier formation stage are apt to receive endosomal traffic.

During the carbohydrate synthesis stage intra-Golgi transport presumably takes place, resulting in the exchange of Golgi resident glycosylation enzymes between cisternae and in the formation of enzyme gradients (Papanikou and Glick 2014). During this stage COPI carriers are thought to bud from some cisternae and fuse with other. Directionality during COPI-dependent intra-Golgi traffic that would result in asymmetric Golgi enzyme distribution may be conferred by continuous lipid remodeling in cisternae, combined with the partition of proteins in favorable lipid environments (Patterson et al. 2008, Day et al. 2013, Papanikou and Glick 2014). Transfer of material from older to younger cisternae is thought to be achieved as older cisternae lose their ability to accept COPI carriers while younger cisternae acquire this ability. This is mostly uncharted territory in *A. nidulans*. It is logical to assume that, while cisternae mature (Pantazopoulou and Penalva unpubl, see also below), at some point the early Golgi becomes competent at accepting COPI vesicles recycling Golgi residents, such as glycosylation enzymes, that are being extracted from a later cisterna en route to the carrier-formation stage (FIG. 2B). The medial Golgi should be part of this stage, when viewed from the cisternal maturation standpoint, because it is an intermediate compartment that has to gradually undergo domain segregation and send early Golgi resident-containing membranes to retrograde pathways. The late Golgi would participate in intra-Golgi traffic if, for example, newly formed late Golgi needed further transformation before it reached the carrier-formation stage or if late Golgi residents had to be sent to earlier cisternae, although these possibilities are clearly hypothetical and should be subject to experimentation. If these assumptions are true, the early, medial and late Golgi, for some period of their lifetimes, would participate in COPI-dependent intra-Golgi exchange and thus would be part of what is

considered the carbohydrate assembly stage. A study of COPI localization (and of glycosylation enzymes) across Golgi subcompartments should be informative on intra-Golgi transport and will be reported elsewhere (Hernandez-Gonzalez, Pantazopoulou and Peñalva unpubl). Tubules observed throughout the Golgi might be evidence of intra-Golgi transport in *A. nidulans*, although we have not determined whether there might be tubule-mediated transport of cargo, resident proteins or both. Rab6 is known to regulate intra-Golgi transport and in *rabCD* cells the *A. nidulans* Golgi is fragmented, perhaps as a consequence of perturbed intra-Golgi pathways (Pantazopoulou and Penalva 2011).

A near-complete functional characterization would require the characterization of lipid components in the Golgi apparatus. Within the Golgi exists the demarcation line of two membrane territories with distinct properties: thin and loose bilayers at the ER and the *cis*-Golgi and thick, rigid bilayers, of negative cytoplasmic charge at the *trans*-Golgi and the plasma membrane (Holthuis and Menon 2014). This transition is mediated by sphingolipid and sterol supply so lipid homeostasis proteins have a central role in it. Lipid homeostasis at the Golgi has important roles in exocytic cargo sorting (Hankins et al. 2015b). Although this is largely an unexplored field in filamentous fungi, the GFP::Lact-C2 reporter binding to phosphatidylserine (PS) recently was used to visualize PS distribution in *A. nidulans* (Penalva 2015, Schultzhaus et al. 2015). In tip cells, when expressed at relatively high levels, this reporter labels Golgi-like cytosolic structures apart from the SPZ suggesting that PS is enriched on the cytosolic face of these cisternae. PS faces the lumen of the ER, while it is translocated at the cytosolic leaflet by the action of flippases at the *trans* Golgi (Hankins et al. 2015a). The *A. nidulans* phospholipid translocases (flippases) DnfA (homologue of yeast Dnf1/2) and DnfB (homolog of yeast Drs2) localize at the SPZ and at cytosolic structures, where they co-localize with the late Golgi marker PH^{OSBP}, with DnfB showing a more prominent localization to the latter compartment (Schultzhaus et al. 2015). Although the flippases were not formally shown to segregate from early Golgi markers, these data suggested that differences in lipid homeostasis regulation and thus lipid content would further differentiate the early from the late Golgi in *A. nidulans*. Non-vesicular lipid traffic mediated by lipid transfer proteins may collaborate with the action of flippases to establish lipid asymmetry in membranes (Panatala et al. 2015). OshA, the *A. nidulans* homolog of yeast Osh1 (a member of the yeast oxysterol binding protein-related proteins that regulate lipid homeostasis and are implicated in non-vesicular transport of sterols [de Saint-Jean et al. 2011], ceramide [Kajiwarra et al. 2014]

and phosphatidylserine [Maeda et al. 2013, Moser von Filseck et al. 2015]), appears to localize in Golgi cisternae, similarly to its yeast counterpart (Levine and Munro 2001), and is reported to co-localize with the late Golgi marker mRFP-TlgB (Buhler et al. 2015).

AN ORGANELLE IN DYNAMIC EQUILIBRIUM

Various observations have suggested that the Golgi apparatus is a dynamic organelle with self-organizing properties, maintained in a dynamic state by continuous input of energy (Jackson 2009). Various lines of evidence indicate that this is also true in *A. nidulans*.

Both early and late *A. nidulans* Golgi compartments are transient (Pantazopoulou and Peñalva unpubl and Pantazopoulou et al. 2014). Late Golgi cisternae have an average lifetime of approximately 2 min (Pantazopoulou et al. 2014). That Golgi cisternae are transient is consistent with the cisternal maturation model. The fact that a fraction of cisternae are labeled by both early and late Golgi markers, as visualized in yeast (Losev et al. 2006, Matsuura-Tokita et al. 2006), also is consistent with Golgi maturation. Work to be reported elsewhere also has revealed that indeed early Golgi matures to late Golgi in *A. nidulans* (Pantazopoulou and Peñalva unpubl).

We have observed that early Golgi cisternae localize close to the apex (similar to the ER and ER exit sites (Pantazopoulou and Penalva 2009, Markina-Inarrainzategui et al. 2013), while late Golgi cisternae seem to be excluded from a subapical region (FIG. 2A) whose dimensions correlate with growth speed. Rapid growth correlates with large area of exclusion (in the range of a few micrometers from the apex), while slow growth correlates with smaller area of exclusion. Why is there exclusion of late Golgi from the most apical region? Because cisternae are transient, we assume that they would be continuously replenished in the cell. Because the late Golgi matures from the early Golgi, we would expect its temporal distance from the ER exit sites to be larger than that of the early Golgi. This means that, if lifetime of early Golgi cisternae was approximately 2 min (we have measured late Golgi cisternae lifetime to be 2 min), late Golgi would appear with more than a 2 min delay after assembly of the early Golgi. A 2 min delay would result in a 2 μ m distance from the apex of the most apical late Golgi cisternae (assembled by input from the apical ER exit sites), in hyphae growing at a routine speed of 1 μ m/min (if there was no transport of late Golgi toward the apex) and this in fact is consistent with the distance we have observed. It would be remarkable if the apical late Golgi exclusion zone reflected maturation. However, other factors, such as different cytoskeleton attachment attributes of the late Golgi in comparison

to the early Golgi, may be responsible for these observations.

Although excluded from the most apical tip region, the late Golgi has a remarkably polarized distribution toward the growing tip that is more pronounced than the polarization displayed by the early Golgi or the ER exit sites markers (Pantazopoulou and Penalva 2009). This suggests that the dynamics of membrane traffic pathways involving the Golgi at the crossroads of endocytosis and exocytosis might reach a different equilibrium at the tip. For example, the late Golgi at the tip might enrich its membranes accepting large-scale recycling traffic from the adjacent subapical endocytic ring.

In agreement with cisternae being transient, blocking the ER exit, by shifting to the restrictive temperature a strain carrying a mutant *sarA^{sar1}* allele that causes thermosensitivity, resulted in early Golgi markers (SedV, RerA) re-localizing to the ER (Hernandez-Gonzalez et al. 2015). Block in Golgi assembly by conditional mutants of the essential early Golgi regulators RabO and SedV resulted in early Golgi markers re-localizing to a haze (Pinar et al. 2013). Interfering with the ER-Golgi interface in all three conditional mutants results in late Golgi disorganization with PH^{OSBP} becoming cytoplasmic and TlgB redistributing to faintly labeled structures, possibly Golgi remnants (Pinar et al. 2013, Hernandez-Gonzalez et al. 2015). Rapid disorganization of the late Golgi as a consequence of a block at the early Golgi assembly is consistent with the late Golgi originating from the early Golgi by maturation.

After treatment with BFA, the *A. nidulans* Golgi collapses into large aggregates in fewer than 5 min. This behavior is characteristic of the Golgi in that neither the ER, labelled by the translocon component Sec63-GFP, or the ER exit sites, labelled by the COPII component Sec23-GFP, apparently respond to BFA addition (Pantazopoulou and Penalva 2009, Markina-Inarrairaegui et al. 2013). In mammalian cells, in the presence of BFA, the TGN mixes with recycling endosomes, a marker of which is Rab11 (Sonnichsen et al. 2000) while Golgi is reabsorbed by the ER (Lippincott-Schwartz et al. 1991). By analogy, prolonged (> 5–10 min and < 30–45 min) BFA treatment in *A. nidulans* results in early Golgi marker SedV redistributing to the ER, while the late Golgi, which matures to RabE^{RAB11}-containing post-Golgi carriers in untreated cells (Pantazopoulou et al. 2014), remains ER-independent (Pantazopoulou and Penalva 2011 and unpubl obs). BFA also leads to a transient apical extension arrest (Pantazopoulou and Penalva 2009). *A. nidulans* hyphae recover spontaneously from BFA treatment (> 45 min to 1 h), resuming growth as the Golgi apparatus reorganizes. The role that Golgi BFA aggregates

may have in the reorganization of the Golgi structure during the recovery is unknown.

Although dynamic the *A. nidulans* Golgi complex does not undergo fragmentation during mitosis (Pantazopoulou and Penalva 2009) (*A. nidulans* mitosis is closed, meaning that the nuclear membrane is not disassembled, although nuclear pore complexes are [De Souza et al. 2004]), unlike what is seen in mammalian cells. In that respect Golgi of filamentous fungi is similar to yeast Golgi.

RAB^{RAB6} AND ACTIN FILAMENTS CONTRIBUTE TO GOLGI ARCHITECTURE AND DYNAMICS

Yeast Ypt6 (human Rab6) is a Golgi-associated Rab GTPase implicated in several Golgi-related retrograde pathways. For example, Ypt6 interacts with subunits of the GARP tethering complex, acting on retrograde transport from the endosomes to the late Golgi (Siniosoglou and Pelham 2001, Bonifacino and Hierro 2011); Rab6 effectors interact with the retromer coat complex implicated in protein recycling from the endosomes to the Golgi (Wassmer et al. 2009); COG8, a nonessential subunit of the intra-Golgi-transport-mediating multisubunit tether COG, becomes essential in the absence of Ypt6 (Whyte and Munro 2001); Rab6 also is implicated in CopI-dependent and independent retrograde intra-Golgi and Golgi-to-ER pathways (Girod et al. 1999, Smith et al. 2009, Storrie et al. 2012). Many golgins (proteins that influence Golgi organization) are known effectors of Rab6 (Ramirez and Lowe 2009). In *A. nidulans* RabC^{Rab6} localizes at the Golgi (Pantazopoulou and Penalva 2011). Subunits of the COG complex also localize at the Golgi and have an impact on polarized growth and protein glycosylation (Gremillion et al. 2014). *A. nidulans rabCΔ* mutant is viable, even though it has major growth defects, both in hyphal morphology and growth rate (Pantazopoulou and Penalva 2011, Penalva et al. 2012). We have shown that *rabCΔ* growth defect correlates with severely altered Golgi architecture. Both late PH^{OSBP}- and early SedV-labeled cisternae in *rabCΔ* are much smaller and more numerous than in the wild type, suggesting that cisternae are fragmented (Pantazopoulou and Penalva 2011). The Golgi apparatus also is severely depolarized. The late Golgi apex-proximal exclusion zone (see above and FIG. 2A) is largely lost. Moreover, BFA treatment of *rabCΔ* cells does not result in collapse of the Golgi into BFA-aggregates, suggesting that this collapse is RabC-dependent. Although these observations established an important role of RabC in Golgi architecture and dynamics, the mechanistic basis of this role is not understood. *rabCΔ* growth defect also correlates with a more prominent SPZ, probably linked to altered secretory carriers'

fusogenicity (Pantazopoulou and Penalva 2011, Penalva et al. 2012). Identification and study of RabC effectors in the future may be instrumental in understanding the multiple roles that RabC exerts on secretion.

Golgi architecture also depends on F-actin in *A. nidulans*, because F-actin depolymerization by Latrunculin B resulted in late Golgi cisternae fragmentation and depolarization and loss of the late Golgi subapical exclusion zone, an effect that overall is similar to the effect *rabCΔ* has on the Golgi (Pantazopoulou and Penalva 2009).

UNLOCKING GOLGI EXIT

The Golgi maturation model for cargo transport predicts that Golgi cisternae eventually dissipate into secretory carriers and carriers bound to the endosomal system.

RabE^{RAB11} and its Guanine nucleotide exchange factor (GEF), *TRAPPII*.—In yeast three Rab GTPases regulate exocytosis, the Ypt31/Ypt32 paralogs (and Rab11 homologs), and Sec4, a homolog of Rab8. Ypt31/32, is involved in Golgi exit and collaborates with PI4P at late secretory compartments to recruit myosin V for polarized transport of secretory carriers (Jedd et al. 1997, Lipatova et al. 2008, Jin et al. 2011, Santiago-Tirado et al. 2011). Myosin V contributes to secretion in *U. maydis* and *A. nidulans* (Zhang et al. 2011, Schuster et al. 2012, Taheri-Talesh et al. 2012). Yeast Sec4 acts downstream of Ypt31/32 (Jedd et al. 1997, Ortiz et al. 2002). Unlike in yeast Sec4 homologs are not essential in *A. niger* (Punt et al. 2001), *A. fumigatus* (Powers-Fletcher et al. 2013) or *A. nidulans* (Pantazopoulou et al. 2014), but the only *A. nidulans* Ypt31/Ypt32 homolog, RabE, is essential (Pantazopoulou et al. 2014). *A. nidulans* GFP-Sec4 co-localizes at the SPZ with GFP-RabE, but GFP-RabE additionally localizes at rapidly moving structures and Golgi-like elements (Pantazopoulou et al. 2014). By co-filming GFP-RabE with the late Golgi marker mRFP-PH^{OSBP}, we found that late Golgi cisternae are transient and that, at the final stage of their life cycle, which is marked by a gradual drop in PH^{OSBP}, they recruit GFP-RabE. Thus PH^{OSBP} and RabE co-localize at late cisternae for approximately 25 s. After this period PH^{OSBP} drops below the detection limit, which is also true for Sec7-GFP, and this coincides with acquirement of long-range motility by GFP-RabE-containing membranes. Thus PH^{OSBP} subsidence coincides with Golgi exit, demarcating the passage from the Golgi apparatus to post-Golgi carriers. GFP-RabE carriers that are loaded with myosin V, one or more kinesins and dynein and whose motility depends on both actin

and microtubules, have been observed in high temporal resolution movies to bombard the SPZ (Pantazopoulou et al. 2014). Motile GFP-RabE carriers are clearly distinct from the similarly motile RabA^{Rab5} early endosomes (Abenza et al. 2009, Pantazopoulou et al. 2014).

But what exactly regulates this transformation at the edge of the Golgi territory? GFP-RabE accumulation at the SPZ depends on BFA for in its presence GFP-RabE becomes cytoplasmic, which suggests that as expected membrane recruitment of RabE depends on functional Golgi. BFA has predictably two targets at the *A. nidulans* Golgi: the two Golgi Arf1 GEFs, that is GeaA, homolog of the early Golgi GBF1/Gea1p and HypB^{Sec7}, homolog of the late Golgi BIG/Sec7p. However, although shifting the conditional late Golgi *hypB5^{sec7ts}* mutant (Yang et al. 2008, Arst et al. 2014) to the restrictive temperature resulted in GFP-RabE relocating from the SPZ to abnormal membrane aggregations in the proximity of the nucleus, it did not mimic the dispersion to the cytoplasm elicited by BFA. This suggests that RabE recruitment to membranes depends on a determinant acting upstream of (or independent of) HypB^{Sec7} and downstream of the early BFA target, the Golgi Arf1-GEF GeaA.

hypA1 is another thermosensitive mutant of the *hyp* collection, found to control hyphal morphogenesis through secretion (Kaminskyj and Hamer 1998, Harris et al. 1999, Shi et al. 2004). HypA is a homolog of Trs120, a subunit of the Golgi-related oligomeric transport protein particle (TRAPP) complex. TRAPP comes in two versions, TRAPPI and TRAPP II, the latter deriving from the former by acquisition of three TRAPP II-specific subunits, Trs120, Trs130 and Trs65 (Sacher et al. 2008). TRAPPI is a demonstrated GEF of Ypt1/Rab1 (GEFs stimulate GDP release to allow binding of GTP by GTPases and determine Rab GTPases membrane recruitment and the subsequent recruitment of Rab effectors). The physiological role of TRAPP II was controversial: Some data pointed at TRAPP II being the GEF of Ypt31, which suggested that conversion of TRAPPI into TRAPP II at the Golgi would result in a transition from an earlier, Ypt1-containing, to a later, Ypt31-containing, compartment (Sacher et al. 2008). However, other studies have showed that in vitro both TRAPPI and II are Ypt1 GEFs (Cai et al. 2008), suggesting that one version of TRAPP might activate Ypt1 at the early Golgi, while the other might activate Ypt1 at the late Golgi. Using genetic, biochemical and microscopy tools, as well as the emerging power of *A. nidulans* cell biology comprehension, this major controversy in the field of Golgi biology was settled with help from hyphal cells. HypA^{Trs120} is essential. Shifting *hypA1* cells to the restrictive temperature relocates RabE from SPZ-associated membranes to the

cytoplasm (like BFA), while it does not affect RabO^{Ypt1} localization, consistent with *hypA1* inactivating a RabE-specific GEF (Pinar et al. 2015). A laborious effort to recover extragenic suppressors of *hypA1* yielded a collection of RabE mutants, of which several were shown to accelerate RabE GDP release (Pinar et al. 2015). These mutants rescued the null *hypAΔ* mutant, and remarkably they also rescued *hypCΔ*, the null mutant of the gene coding for Trs130, another essential TRAPPII-specific subunit. These mutants on the other hand did not rescue a null mutant of a TRAPP core subunit (i.e. common for TRAPPI and TRAPPII) or the null *rabOΔ* mutant, suggesting that they specifically bypass TRAPPII. TRAPPII subunits were further shown to associate with RabE in vivo but not with RabO. In vitro TRAPPII exhibited GEF activity on both RabO and RabE, suggesting that TRAPPI activity is retained in TRAPPII, perhaps because the formation of a RabE-specific binding site in TRAPPII does not perturb the RabO binding site on TRAPP (Pinar et al. 2015). TRAPPII-specific Trs120-GFP colocalizes with RabE at the late Golgi but, contrary to RabE, is largely released before carriers arrive at the SPZ, consistent with TRAPPII acting upstream of RabE at the late-Golgi to post-Golgi transition (Pinar et al. 2015).

It is plausible that recruitment of TRAPPII to the late Golgi is independent of HypB^{Sec7} and this is why RabE remains associated with membranes in the *hypB5* mutant. However, it is expected that *hypB5* would affect the exit from the Golgi toward the endosomal system, in that all known adaptors of cargo destined to the endosomes are effectors of Arf1 at the late Golgi, with HypB^{Sec7} being the late Golgi Arf1 GEF. Shifting *hypB5* at the restricting temperature consistently blocks the apex-wise motility of RabE carriers with RabE membranes accumulating intracellularly. At those accumulations RabE membranes are abnormally associated with RabA^{Rab5} membranes (RabA^{Rab5} is a marker of early endosomes [Abenza et al. 2009]) (Pantazopoulou et al. 2014), perhaps because HypB^{Sec7} perturbation impedes cargo sorting at the late Golgi/TGN, that would result in unresolved compartments of mixed endosomal/secretory identity.

The Arf1 Golgi GEFs, GeaA and HypB^{Sec7}.—The fact that treatment with BFA (predictably blocking GeaA and HypB, as explained above) redistributes RabE from membranes to the cytoplasm, while *hypB5* shift at the restrictive temperature does not impede RabE membrane association, would suggest that it is the early Golgi Arf1 GEF, GeaA, that is indispensable for RabE membrane association. This is perhaps because GeaA has a more central role on Golgi integrity than HypB^{Sec7} that might play a more specific role on Golgi exit used by specialized cargoes.

The roles that two distinct, universally conserved in eukaryotes, Golgi GEFs for Arf1 play are not clear (Mouratou et al. 2005). Each Arf1 GEF is present either at the early or late Golgi. It has been suggested that these large (≈ 1500 – 2000 aa), multidomain proteins themselves can contact specific protein interactors, modifying the outcome of Arf1 activation at each Golgi subcompartment. Yeast late Golgi Sec7, for example, has been shown to regulate and/or integrate feedbacks from multiple master Golgi regulators (Arf1, Arf1, Ypt1, Ypt31/32), resulting in coordination of Golgi functions, especially of Golgi entry and exit (McDonold and Fromme 2014). Yeast early Golgi Gea1p has been shown to interact with a subunit of CopI (Deng et al. 2009), an Arf1 effector that has roles in intra-Golgi and Golgi-to-ER traffic. Apart from their largely unexplored roles, the specific membrane recruitment mechanism of Golgi Arf1 GEFs (they are peripheral membrane proteins) to specialized Golgi subcompartments is not understood (Wright et al. 2014).

Both Arf1-GEFs in *A. nidulans*, HypB^{Sec7} and GeaA, are virtually essential (Arst et al. 2014). Using genetics once again, extragenic suppressors of the conditional late Golgi Arf1 GEF mutant *hypB5* were sought (Arst et al. 2014). Among lots of intragenic suppressors, we recovered a single extragenic one that carries a mutation in the early Golgi Arf1 GEF, GeaA. The *geaA1* mutant allele rescued *hypBΔ*. In silico analysis of the similarity of proteins in the GBF/GeaA and BIG/Sec7 subfamilies, showed that the amino acid substitution in GeaA1 lies in a GBF/Gea-specific (not found in BIG/Sec7) tripeptide motif. Remarkably the suppressor mutation substantially shifted the localization of GeaA1-GFP from the early Golgi to the apical plasma membrane and to a SPZ-resembling accumulation beneath it (Arst et al. 2014). The mechanism by which GeaA1 bypasses HypB^{Sec7} essential functions remains unexplored. However, toleration of such a break in Golgi Arf1 GEFs asymmetry was unprecedented in eukaryotes. Answering how Golgi subcompartments lacking a master late Golgi regulator are maintained and maintain filamentous fungal growth most probably would provide new insights on Golgi function in the future.

ACKNOWLEDGMENTS

I thank Miguel Peñalva for constructive discussions and for critically reading the manuscript. I thank Richard Howard, Michelle Momany and Jaclyn Dee (and of course their collaborators) for kindly letting me use their electron microscopy data. This work was supported by Ministerio de Economía y Competitividad (Spain) Grant BIO2012-30965 and Comunidad de Madrid Grant S2010/BMD-2414 to Miguel Peñalva.

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