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Architecture and development of the *Neurospora crassa* hypha – a model cell for polarized growth

Meritxell RIQUELME[a,*], *Oded YARDEN*[b,*], *Salomon BARTNICKI-GARCIA*[a], *Barry BOWMAN*[c], *Ernestina CASTRO-LONGORIA*[a], *Stephen J. FREE*[d], *Andre FLEIßNER*[e], *Michael FREITAG*[f], *Roger R. LEW*[g], *Rosa MOURIÑO-PÉREZ*[a], *Michael PLAMANN*[h], *Carolyn RASMUSSEN*[i], *Corinna RICHTHAMMER*[j], *Robert W. ROBERSON*[k], *Eddy SANCHEZ-LEON*[a], *Stephan SEILER*[j], *Michael K. WATTERS*[l]

[a]*Center for Scientific Research and Higher Education of Ensenada – CICESE, Ensenada Baja California 22860, Mexico*
[b]*Department of Plant Pathology and Microbiology, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel*
[c]*Department of Molecular, Cell and Developmental Biology, University of California, Santa Cruz, CA 95064, USA*
[d]*Department of Biological Sciences, University at Buffalo, Buffalo, NY 14260, USA*
[e]*Institut für Genetik, Technische Universität Braunschweig, 38106 Braunschweig, Germany*
[f]*Department of Biochemistry and Biophysics, Center for Genome Research and Biocomputing, Oregon State University, Corvallis, OR 97331-7305, USA*
[g]*York University, Toronto, Ontario M3J 1P3, Canada*
[h]*School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO 64110, USA*
[i]*University of California, San Diego, Cell and Developmental Biology, 9500 Gilman Dr., La Jolla, CA 92093-0116, USA*
[j]*Department of Molecular Microbiology and Genetics, Institute of Microbiology and Genetics, Georg-August-Universität, D-37077 Göttingen, Germany*
[k]*School of Life Sciences, Arizona State University, Tempe, AZ 85287, USA*
[l]*Department of Biology, Valparaiso University, Valparaiso, IN 46383-4543, USA*

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ABSTRACT

Neurospora crassa has been at the forefront of biological research from the early days of biochemical genetics to current progress being made in understanding gene and genetic network function. Here, we discuss recent developments in analysis of the fundamental form of fungal growth, development and proliferation – the hypha. Understanding the establishment and maintenance of polarity, hyphal elongation, septation, branching and differentiation are at the core of current research. The advances in the identification and functional dissection of regulatory as well as structural components of the hypha provide an expanding basis for elucidation of fundamental attributes of the fungal cell. The availability and continuous development of various molecular and microscopic tools, as utilized by an active and co-supportive research community, promises to yield additional important new discoveries on the biology of fungi.

* *Corresponding authors.*
E-mail address: riquelme@cicese.mx
1878-6146/$ – see front matter
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Introduction

Neurospora crassa - a model going strong

Our understanding of the morphogenesis of filamentous fungi is progressing rapidly (with >15 000 publications in just the last 5 y). The wealth of genetic information, availability of mutants and the progress made in live imaging techniques, coupled with biochemical analysis, have significantly contributed to the progress made in understanding one of the most characteristic and fundamental forms of fungal growth, development and proliferation – the hypha. Along with neurons and pollen tubes, hyphae are the most highly polarized cell forms known (Palanivelu & Preuss 2000; Borkovich *et al.* 2004; Harris 2006; Ischebeck *et al.* 2010). On the one hand, much has been discovered about the role and function of hyphal elements that are shared with many other eukaryotic cell types, albeit in the context of a syncytium. On the other hand, many structures and functions unique to filamentous fungi have now been identified and analyzed. Thus, the accumulating information, along with the technological advances enhancing our capabilities of probing and analyzing both existing and new directions, make the compilation of this review timely. Featuring *N. crassa*, we intend this review to serve as an updated resource and a source of ideas for future studies on the fungal filament. Furthermore, the increased interest in fungal pathogens of humans, animals and plants, along with the use of filamentous fungi in biotechnology and bioprospecting warrants the in-depth understanding of the hyphal filament as the fundamental unit in these organisms.

Neurospora crassa has been an excellent model organism for eukaryotic genetics and biochemistry and one of the workhorses for fungal cell biology research. While *Saccharomyces cerevisiae* is often referred to as a good representative of the Fifth Kingdom, it has become increasingly apparent that - despite its virtues - the yeast cell represents only a minor fraction of the fungal kingdom in many morphological and biochemical aspects. Most fungi have a highly branched filamentous morphology and occupy a much broader spectrum of habitats. The rapid (~4 mm hr^{-1}) filamentous growth habit of *N. crassa* is the result of a strongly polarized mechanism culminating in the biogenesis of the tubular cell wall. Seven decades of pioneering research on the biology of the hypha performed with this model organism (Beadle & Tatum 1945; Garnjobst & Tatum 1967; Collinge & Trinci 1974; Vollmer & Yanofsky 1986; Metzenberg & Glass 1990; Yarden *et al.* 1992; Plamann *et al.* 1994; Steinberg & Schliwa 1995; Seiler *et al.* 1997; Riquelme *et al.* 1998; Davis 2000; Perkins *et al.* 2001; Seiler & Plamann 2003; Gavric & Griffiths 2003), have proven *N. crassa* to be a rewarding model fungus for experimental work – work that continues today in more than 30 laboratories around the world. Extensive work has been performed, utilizing *N. crassa*, on genome defence, DNA repair and recombination, on light and circadian regulation as well as on mitochondrial protein import and biogenesis, but because of the scope of this article we refer readers interested in this subject to recent reviews and genome-wide studies that describe the relevant findings and address the challenges in these fields (Galagan & Selker 2004; Ninomiya *et al.* 2004; Neupert & Herrmann 2007; Jinhu & Yi 2010; Chen *et al.* 2010; Vitalini *et al.* 2006; Smith *et al.* 2010; Chen *et al.* 2009; Borkovich *et al.* 2004).

The entire community of fungal biologists has benefited from useful resources derived from the Functional Genomics and Systems Biology Project, a project promoted by members of the Neurospora community that culminated in the publication of the *N. crassa* genome draft sequence (Galagan *et al.* 2003; Borkovich *et al.* 2004; Dunlap *et al.* 2007). Some valuable tools include a collection of single-gene deletion mutants (Colot *et al.* 2006), as well as expression and tiling microarrays (Greenwald *et al.* 2010; Hutchison *et al.* 2009; Kasuga & Glass 2008), and single nucleotide polymorphism data for widely used strains (Lambreghts *et al.* 2009). The publication of the first high-quality draft genome of a filamentous fungus was just the beginning. In fact, ~200 fungal genome sequences will soon be available (for details see http://fungalgenomes.org/wiki/Fungal_Genome_Links). This number will greatly increase in the near future as high-throughput sequencing allows affordable sequencing and *de novo* assembly of fungal genomes (Nowrousian 2010). In addition to genetics-based developments and tools, techniques that have progressed our abilities to study the cell biology of *N. crassa* have also evolved. Fluorescent protein (FP) labelling was successfully developed for *N. crassa* separately by two different labs in 2001 and 2002 (Freitag *et al.* 2001; Fuchs *et al.* 2002), and made widely available to the Neurospora community in 2004 (Freitag *et al.* 2004). Currently, an ever-increasing number of strains with fluorescently labelled proteins (Table 1) are readily available from the Fungal Genetics Stock Center (http://www.fgsc.net/).

By providing a critical and current evaluation of research on one of the most advanced model systems useful to all researchers studying filamentous fungi, we hope to stress opportunities for future research directions and identify important challenges.

The hyphal lifestyle encompasses multiple morphological structures

The ability to form polarized cell types is not only a fundamental property of filamentous fungi, but is also one of the key attributes that contributes to their success in inhabiting beneficial niches and/or avoiding detrimental ones. As such, the development of hyphae is one of the bases for fungal proliferation. In many cases, hyphal development can be a prerequisite for the formation of additional cell types that, along with hyphae, are involved in growth, development and propagation.

Amongst at least 28 distinct morphological cell types described in *Neurospora crassa* (Bistis *et al.* 2003), more than six can be designated as hyphae. These forms of hyphae encompass both asexual and sexual development of this fungus. The hyphal cell types described include (for more details see Bistis *et al.* 2003): Leading hypha (wide, fast growing with subapical branching; Robertson 1965); Trunk hypha (in the colony interior); Fusion hypha and conidial anastomosis tubes hypha (bridge between hypha and between conidia; Glass *et al.* 2004; Roca *et al.* 2005); Aerial hyphae (growing away from the medium and required for macroconidiation); Enveloping (or ascogonial investing) hyphae (engulf the ascogonium; Read

Table 1 – Compendium of the genes and their locus tags included in this review and for which the corresponding protein tagged with fluorescent proteins have provided their cellular localization and confirmed their role in hyphal morphogenesis in *N. crassa*.

Gene Name	Locus	Role	Localization	Reference
Cell wall				
gs-1	NCU04189	*Glucan Synthase Regulator:* Putative regulator of cell wall glucan synthase enzyme	Accumulates at hyphal apex at the outer macrovesicular stratum of Spk, surrounding the inner core of chitin synthase containing microvesicles	Verdin *et al.* 2009
chs-1	NCU03611	Probably involved in cell wall chitin biosynthesis	Localized at Spk core of active growing hyphae, during septum development, and spherical and enlarged vacuolar system	Sanchez-Leon *et al.* in press
chs-2	NCU05239	Not essential for cell wall chitin content	Accumulates during cell wall septum development[a]	Riquelme lab[a]
chs-3	NCU04251	Probably involved in cell wall chitin biosynthesis	Localized at Spk core of active growing hyphae, during septum development, and spherical and enlarged vacuolar system	Riquelme *et al.* 2007
chs-4	NCU09324	Probably involved in septum cell wall chitin biosynthesis.	Accumulates during cell wall septum development[a]	Riquelme lab[a]
chs-5	NCU04352	Probably involved in cell wall chitin biosynthesis	Localized at Spk core of active growing hyphae	Riquelme lab[a]
chs-6	NCU05268	Probably involved in cell wall chitin biosynthesis	Localized at Spk core of active growing hyphae, during septum development, and spherical and enlarged vacuolar system	Riquelme *et al.* 2007
chs-7	NCU05350	Probably involved in septum cell wall chitin biosynthesis	Accumulates during cell wall septum development[a]	Riquelme lab[a]
Cytoskeleton genes				
bml	NCU04054	Beta-tubulin	Mts of cortical and central cytoplasmic hyphal regions and in the cytoplasm of young apical hyphal compartments, and MTOC.	Freitag *et al.* 2004; Mouriño-Pérez *et al.* 2006
fim	NCU003992	Fimbrin, an actin-binding protein	Small patches in cortical cytoplasm. Flanking the developing septa.	Delgado-Alvarez *et al.* 2010
tpm-1	NCU001204	Tropomyosin, an actin binding protein	Localized at Spk, actin cables and mature septa	Delgado-Alvarez *et al.* 2010
arp-3	NCU001756	Subunit of the Arp2/3 complex	Small patches in cortical cytoplasm. Flanking the developed septa.	Delgado-Alvarez *et al.* 2010
Nuclei				
dbf-2	NCU09071	NDR protein kinase that functions as a link between Hippo and glycogen metabolism pathways.	Localized at the nucleus	Dvash *et al.* 2010
hh1	NCU06863	Histone	Unevenly distributed in nuclei and also localized on stable foci.	Freitag *et al.* 2004
Hpo	NCU04018	Heterochromatin protein HP1. Essential for DNA methylation.	Heterochromatic foci in nuclei	Freitag *et al.* 2004; Freitag & Selker 2005; Bowman *et al.* 2009
son-1	NCU04288	Nucleoporin, Nuclear pore complex marker	Localized at nuclear envelope in a discontinued manner and nuclear pores throughout nuclear cycle	Roca *et al.* 2010

Endoplasmic reticulum				
grp-78	NCU03982	ER-associated HSP. Facilitates protein folding in the ER.	Nuclear envelope and associated membranes. ER.	Bowman *et al.* 2009
dpm	NCU07965	Dolichol-phosphate mannosyltransferase	Nuclear envelope and associated membranes. ER.	Bowman *et al.* 2009
Vacuoles				
vma-1	NCU01207	Subunit A of vacuolar ATPase	Vacuolar membrane and unidentified organelle membrane	Bowman *et al.* 2009
vam-3	NCU06777	Vacuole-associated SNARE protein	Localized as a dense tubular network. Small vesicles and spherical vacuoles at distal cell regions.	Bowman *et al.* 2009
vma-5	NCU09897	Subunit C of vacuolar ATPase	Unidentified organelle membrane	Bowman *et al.* 2009
Mitochondria				
arg-4	NCU10468	Mitochondrial acetylornithine-glutamate transacetylase. Arginine biosynthesis	Localized in mitochondria.	Bowman *et al.* 2009
Golgi				
vps-52	NCU05273	Component of Golgi body-associated retrograde protein complex	Putative late Golgi compartment	Bowman *et al.* 2009
Calcium Transporters				
nca-1	NCU03305	Ca^{2+}/H^{+}-ATPase	Localized at nuclear envelope and associated membranes. Endoplasmic reticulum	Bowman *et al.* 2009
nca-2	NCU04736	Ca^{2+}/H^{+}-ATPase	Plasma and Vacuolar membrane. Localized as a dense tubular network. Small vesicles and spherical vacuoles at distal cell regions.	Bowman *et al.* 2009
nca-3	NCU05154	Ca^{2+}/H^{+}-ATPase	Plasma and Vacuolar membrane. Vacuolar network, large spherical vacuoles.	Bowman *et al.* 2009
cax	NCU07075	Ca^{2+}/H^{+} exchange protein	Vacuolar compartments. Localized as a dense tubular network. Small vesicles and spherical vacuoles at distal cell regions. Unidentified organelle membrane.	Bowman *et al.* 2009
Exocyst				
sec-3; sec-5; sec-6; sec-8; sec-15; exo-84; exo-70	NCU09869 NCU07698 NCU03341 NCU04190 NCU00117 NCU08012 NCU06631	Component of the exocyst octameric protein complex	Localize primarily as a crescent adjacent to the cell surface at the hyphal dome.	Riquelme & Freitag[a]
Polarity				
bem-1	NCU06593	MAP kinase activator, functions as a scaffold linking MAP kinase signalling and polarity Establishing.	Growing tips of hyphae and germlings, and localize around the septal pore.	Fleissner 2010[a]
bni-1	NCU01431	Involved in actin organization.	Localized at the Spk, apical tips and constricting rings of forming septum	Justa-Schuch *et al.* 2010
cla-4	NCU00406	Unknown function. Putative involved in septum formation	Localized at incipient septation sites and cortical rings. Also localized at Spk and the apical dome.	Justa-Schuch *et al.* 2010
cot-1	NCU07296	NDR kinase, essential for polar cell extension	Localized as punctuate structures evenly distributed through the hyphae (immunolocalization)	Seiler *et al.* 2006
lrg-1	NCU02689	RHO-1-specific GAP. Involved in coordinating apical tip growth	Localized at apical tips as caps and during septation around the septal pore.	Vogt & Seiler 2008

(*continued on next page*)

Table 1 – (*continued*)

Gene Name	Locus	Role	Localization	Reference
mak-2	NCU02393	MAP-kinase2, required for cell fusion	Localized at CAT tips of germlings undergoing chemotropic attraction	Fleißner *et al.* 2009b
pod-6	NCU02537	NDR kinase	Localized as punctuate structures evenly distributed through the hyphae (immunolocalization)	Seiler *et al.* 2006
spa-2	NCU03115	Subunit of the polarisome complex	Localized at the apex of germ tubes, and partially colocalized at Spk.	Araujo-Palomares *et al.* 2009
Septation				
bud-3	NCU06579	Involved in septum formation. Rho-4-specific GEF and putative landmark protein.	Localized at cortical rings prior and during septum development. Also around septal pore after septum is formed. Plasma membrane, septum, and cytoplasm of germlings.	Seiler & Justa-Schuch 2010; Justa-Schuch *et al.* 2010
bud-4	NCU00152	Involved in septum formation and putative landmark protein.	Localized at cortical rings prior and during septum development. Also around septal pore after septum is formed.	Justa-Schuch *et al.* 2010
cdc-12	NCU03795	Unknown function. Putatively involved in septum formation	Localized at incipient septation sites and cortical rings.	Justa-Schuch *et al.* 2010
rgf-3	NCU02131	Involved in septum formation. Rho-4-specific GEF landmark protein	Localized at cortical rings prior and during septum development. Plasma membrane, septum, and cytoplasm of germlings.	Justa-Schuch *et al.* 2010
rho-4	NCU03407	Rho GTPase. Putative marker at mature septum.	Localized at cortical rings prior and during septum development. Also around septal pore after septum is formed.	Rasmussen & Glass 2007; Justa-Schuch *et al.* 2010
so	NCU02794	Contributes to septal plugging.	Localized at particulate complexes at tips of CATs undergoing chemotropic attraction. Localizes at septal plugs of injured hyphae.	Fleißner *et al.* 2009b; Fleißner & Glass 2007
Hyphal fusion				
prm-1	NCU09337	Pheromone-regulated membrane protein 1-like, involved in cell fusion events.	Localized at vacuolar, ER-like compartments, plasma membrane, punctate structures and hypha fusion points.	Fleißner *et al.* 2009a

a Unpublished results.

1983; 1994); Trichogyne (exhibits a positive tropism towards cells of opposite mating type; Bistis 1981); Ascogenous hyphae (contain nuclei of both mating types; Raju 1980, 1992).

Many structural and regulatory elements required for, or involved in, hyphal development have been identified over the years, *via* classical genetics analysis of morphological mutants, molecular and biochemical approaches, and advanced microscopy.

Tip growth

Establishment and maintenance of hyphal polarity

The ability of fungi to generate polarized cells with a variety of shapes reflects precise temporal and spatial control over the formation of polarity axes. Hyphal growth requires establishment of a stable axis of polarization during spore germination and maintenance of this axis during tip extension (Momany 2002). A new axis of polarity is also established in a previously silent area of the hyphal subapex during branch formation. Although it is generally assumed that the basal eukaryotic polarity machinery (Nelson 2003) is used during both germination as well as tip extension, the regulation and specific use of the morphogenetic machinery in these two polarization events is only poorly understood. During polarized growth, cell surface expansion is mostly restricted to a defined area, the hyphal tip. One school of thought holds that the establishment and maintenance of polarity involves (i) delimiting the growth site by cortical markers or intrinsic/external polarity cues, (ii) the transduction and amplification of this signal to the cytoskeleton, primarily mediated by small Rho and Ras-type GTPases, (iii) organizing the cytoskeleton and secretory apparatus towards the growing apex, and (iv) restricting the site of vesicle-plasma membrane fusion to the cell apex. Another school of thought maintains that polar growth is not primarily governed by cortical targets but is generated internally by the displacement of an organizer of vesicle traffic, i.e., the Spitzenkörper acting as a vesicle supply center (Bartnicki-Garcia *et al.* 1989).

The comparison of *Saccharomyces cerevisiae* morphogenetic data with available results gathered from various filamentous fungi (both asco- and basidiomycetes) has revealed that a core of 'polarity factors' is conserved between unicellular and filamentous fungi (Wendland 2001; Borkovich *et al.* 2004; Harris 2006; Garcia-Pedrajas *et al.* 2008; Fischer *et al.* 2008; Harris *et al.* 2009). However, subtle differences in the 'wiring' of these conserved components and the presence of additional proteins that are absent in yeast may be responsible for the greater morphogenetic potential of filamentous fungi (Seiler & Plamann 2003; Malavazi *et al.* 2006; März *et al.* 2010). Strikingly, filamentous fungal proteins resemble homologues of higher eukaryotes more than those of budding yeast.

Cortical landmarks, Ras/Rho GTPases modules and the polarisome

Extensive genetic analyses have provided a fairly detailed understanding of the molecular mechanisms that underlie the axial and bipolar budding patterns in budding yeast (summarized in Park & Bi 2007). The axial pattern is determined by the cell wall protein Axl2p and its association with the septin-interacting proteins Bud3p and Bud4p. For the bipolar pattern, the paralogous cell wall proteins Bud8p and Bud9p and the membrane anchors Rax1p and Rax2p serve as distal and proximal pole markers, respectively. This positional information is relayed to the Ras-like Bud1p/Rsr1p GTPase module *via* the guanine nucleotide exchange (GEF) factor Bud5p and results in localized activation of the Rho-like GTPase Cdc42p, which acts *via* multiple effectors, such as the polarisome and exocyst complexes to recruit components of the morphogenetic machinery to the specified bud site of the yeast cell. The annotation of multiple genomes of filamentous ascomycetes has revealed the presence of several genes homologous to the *Saccahromyces cerevisiae* bud site selection machinery (Borkovich *et al.* 2004; Harris *et al.* 2009; Seiler & Justa-Schuch 2010). However, the extent to which this system is conserved in the highly polarized filamentous fungi remains unknown. While the presence of the paralogous Bud8p/Bud9p markers is restricted to close homologues of *S. cerevisiae* (e.g., *Ashbya gossypii*; Wendland & Walther 2005), the *Neurospora crassa* homologues of the Bud3p–Bud4p complex are not required for polarized hyphal growth, but are critical for specifying the site of septum formation (Justa-Schuch *et al.* 2010; Si *et al.* 2010). Moreover, the presence of a signalling module homologous to Rsr1p–Bud2p–Bud5p in the *N. crassa* is currently questionable. Over-expression of the *N. crassa* ras-related protein KREV-1 induces a random budding pattern in *S. cerevisiae*, implicating it as a potential homologue of the Ras-type GTPase Rsr1p (Ito *et al.* 1997). However, neither loss of function nor dominant mutations result in vegetative defects in *N. crassa*. Instead, *krev-1* mutants are defective in sexual fruiting, body maturation and ascosporogenesis, and it remains currently unclear, if KREV-1 is a functional Rsr1p homologue. Conditional *cdc-25* (the closest *N. crassa* homologue of Bud5p; Seiler & Plamann 2003) mutants display cell polarity defects in subapical regions of the hypha, generating chains of spherical cells after transfer to resctrictive conditions. Loss of function mutations are lethal and only strains carrying heterokaryotic deletions can be maintained, indicating that Ras-type GTPases are central for polarity establishment. In addition to KREV-1, the *N. crassa ras* family of monomeric GTPases is represented by *band/ras-1* and *smco-7/ras-2* (Altschuler *et al.* 1990; Kana-uchi *et al.* 1997). RAS-1 is involved in light and circadian signalling based on analysis of the dominant *band* allele of *ras-1*, *ras-1*^{bd}; (Belden *et al.* 2007), but other morphological functions are likely, yet to date have not been analyzed. RAS-2 has a general impact on hyphal morphology. *ras-2* mutants are characterized by slow growth, increased branching and decreased aerial hyphae and conidia formation (Kana-uchi *et al.* 1997). Two additional ras-related genes are present in the genome (NCU01444 and NCU06111), but functional data concerning these are still unavailable.

Apart from the six Rho GTPase subfamily members RHO-1 to RHO-4, CDC-42 and RAC, the genome of *N. crassa* encodes seven putative RhoGEFs (six of them belonging to the classical Dbl homology family, one with similarity to CZH-type characterized by a docker domain), ten RhoGAPs and one RhoGDI. It is obvious that the multitude of regulators allows sophisticated orchestration of Rho GTPase functions, and as phylogenetic deduction of specificity is limited to few well-conserved

regulators with known targets in other organisms, the experimental determination of regulator specificity in conjunction with detailed phenotypic analysis of mutants is vital to define and characterize the signalling modules and their interconnection. No functional data are currently available for RHO-2, RHO-3 and the RAC in *N. crassa*.

In a large-scale genetic screen to identify conditional mutants defective in cell polarity, mutations of *cdc-42*, its putative GEF *cdc-24* and the presumably interacting scaffold protein *bem-1* resulted in phenotypes implicating these factors in establishment and maintenance of cell polarity, possibly through a role in regulation of actin organization as judged from close resemblance of some of the conditional actin mutants (Seiler & Plamann 2003). The RHO-1-specific GAP LRG-1 is the only RhoGAP characterized so far in *N. crassa* (Vogt & Seiler 2008). Like *rho-1*, *lrg-1* is an essential gene. While functional RHO-1 is necessary for establishment of polarity and homokaryotic deletion mutants germinate isotropically, lack of negative regulation of the GTPase in conditional *lrg-1* mutants leads to the development of pointed, needle-like tips and cessation in tip elongation accompanied by excessive subapical hyperbranching. When LRG-1 function is compromised, putative RHO-1 downstream effectors including β-1,3-glucan synthase, the cell wall integrity MAP kinase pathway and the actin cytoskeleton are misregulated.

The polarisome is important for determining cell polarity, and functions under the control of Cdc42p and other Rho GTPases as the focal point for formin-dependent polymerization of actin monomers into filaments. Homologues of the yeast polarisome components Spa2p, Bud6p and Bni1p were studied in various filamentous fungi, including *N. crassa* (Harris *et al.* 1997; Sharpless & Harris 2002; Virag & Harris 2006; Carbó & Pérez-Martín 2008; Köhli *et al.* 2008; Leeder & Turner 2008; Meyer *et al.* 2008; Araujo-Palomares *et al.* 2009; Justa-Schuch *et al.* 2010; Jones & Sudbery 2010). In *N. crassa* hyperbranching, irregular growth and altered hyphal morphology are the characteristic features of strains lacking an intact polarisome specified by mutations in *spa-2* and/or *bud-6*, while deletion of the sole formin gene *bni-1* is lethal. BNI-1 localizes to the Spitzenkörper of growing hyphal tips and the forming septum, the latter is consistent with a function in contractile acto-myosin ring formation during septum constriction (Justa-Schuch *et al.* 2010). In contrast, SPA-2 localizes exclusively at the hyphal tip of *N. crassa* (Araujo-Palomares *et al.* 2009), suggesting functionally distinct polarisome subcomplexes in *N. crassa* during septation and apical tip extension (Virag & Harris 2006; Leeder & Turner 2008; Araujo-Palomares *et al.* 2009; Justa-Schuch *et al.* 2010). Moreover, the few existing studies on polarisome components in filamenous fungi confirm a highly dynamic and growth dependent behaviour of the polarisome (Köhli *et al.* 2008; Jones & Sudbery 2010) and may further suggest differences in the localization of the component between species. For example, SPA-2 forms a cup-like crescent in *Aspergillus niger* (Meyer *et al.* 2008), while in *Aspergillus nidulans* it is visualized as a bright spot at the tip (Virag & Harris 2006). In *N. crassa,* SPA-2 adopts the shape of an open hand fan with a brighter spot at the base (Araujo-Palomares *et al.* 2009). The observed dissimilarities are intriguing and more studies are required to disclose the functional significance for such differences.

General signalling pathways during cell polarization

One of the best characterized signalling modules involved in regulation of cell polarity is the pathway containing the NDR kinase COT-1 pathway, which interacts with two co-activator proteins of the MOB-2 group and is regulated by the upstream kinase POD-6 (Yarden *et al.* 1992; Seiler *et al.* 2006; März *et al.* 2009). COT-1 and POD-6 have been shown to colocalize at sites of growth along the plasma membrane and the septum and in the cytosol (Gorovits *et al.* 2000; Seiler *et al.* 2006). *cot-1*, *pod-6* and *mob-2* single and double mutants exhibit arrest of hyphal tip extension, marked subapical hyperbranching and altered cell wall and actin organization (Yarden *et al.* 1992; Gorovits *et al.* 2000; Seiler & Plamann 2003; März *et al.* 2009; Ziv *et al.* 2009). Suppression of COT-1 pathway defects occurs through mutations in *gul-1* (Terenzi & Reissig 1967; Seiler *et al.* 2006), a naturally polymorphic protein that has been implicated in the maintenance of cell wall integrity, RNA binding, and protein phosphatase-associated functions in budding and fission yeasts (Matsusaka *et al.* 1995; Uesono *et al.* 1997). In addition, mutations in components of the dynein/dynactin complex (Plamann *et al.* 1994) and mutations or environmental conditions impairing protein kinase A (PKA) activity also suppress COT-1 pathway defects (Gorovits & Yarden 2003; Seiler *et al.* 2006, März *et al.* 2008). Interestingly, there is evidence for links between the COT-1 and RHO-1 signalling pathways. Mutations in *cot-1* pathway genes and in *lrg-1* share the phenotypic characteristics of arrested tip extension and massive hyperbranching, and *cot-1*:*lrg-1* double are synthetic lethal (Seiler & Plamann 2003; Vogt & Seiler 2008). As implied above, PKA activity can be decisive in polar growth. Its hyperactivation by mutation of the regulatory subunit of PKA, *mcb*, results in complete loss of polarity during germination and along growing hyphae (Bruno *et al.* 1996). This abolished negative regulation of PKA activity in *mcb* can be counteracted by decreasing cAMP levels through suppressive mutation of the adenylate cyclase gene *cr-1*. Thus, PKA activity in *N. crassa* clearly promotes apolar growth (and in a manner which is coordinated with regulation of carbon source utlization; Ziv *et al.* 2008), which contrasts the situation in budding yeast, but is similar to animal cells and other filamentous fungi.

Ca^{2+} signalling, which is involved in the regulation of multiple cellular processes (Zelter *et al.* 2004) is also required for hyphal elongation. An internal tip-high Ca^{2+} gradient is required for tip growth (Jackson & Heath 1993; Torralba & Heath 2001). Creation of the tip-high Ca^{2+} gradient can be mediated by mechanosensitive Ca^{2+} permeable channels (Levina *et al.* 1995), based on evidence that mechanosensitive Ca^{2+} permeable channels are tip-localized and mediate Ca^{2+} influx during hyphal tip growth in the oomycete *Saprolegnia ferax* (Garrill *et al.* 1993) and pollen tubes of lilly (Dutta & Robinson 2004). In *N. crassa*, the mechanosensitive channels are not tip-localized (Lew 1998), nor is there tip-localized Ca^{2+} influx at the tip during growth (Lew 1999). In hindsight, this may not be surprising. Various organisms are adapted to specific environs. An oomycete like *S. ferax* (or a pollen tube) grows in freshwater (or a pollination tract) with an assured supply of external Ca^{2+}. A fungus like *N. crassa* is adapted for a wider range of environs, including aerial growth. Thus, it makes sense that it would utilize an internal, protected mechanism

for creating the tip-high Ca^{2+} gradient required for hyphal growth (Silverman-Gavrila & Lew 2000). And in fact, the ion channel responsible for generating the tip-high Ca^{2+} gradient is an endomembrane-located IP_3-activated Ca^{2+} channel (Silverman-Gavrila & Lew 2001; 2002) possibly activated by a membrane-localized phosholipase C (Silverman-Gavrila & Lew 2003).

Another aspect little explored as yet, is the roles of ion transporters in the growth and development of the fungus. As one example, mid1 has been identified as a stretch-activated channel in fungi (Kanizaki *et al.* 1999). In *Saccharomyces cerevisiae*, the knockout mutant dies during mating (a phenotype recovered when the medium is replete with Ca^{2+}; Iida *et al.* 1994). In *N. crassa*, mating is unaffected. Instead, the knock mutant exhibits poor growth and defective plasma membrane transport, likely due to disruption of Ca^{2+} homeostasis (Lew *et al.* 2008). In *Candida albicans*, the lesion appears to be in thigmotropism (Brand *et al.* 2007). Thus, it is possible that the genomic map may not always correspond directly to the phenotypic terrain of growth and development. Only more research will clarify the genotype to phenotype connection.

There are many open questions concerning Ca^{2+} as a morphogen and its role in polarity. How is Ca^{2+} supply to the tip regulated? What is the relation between the tip-high cytoplasmic Ca^{2+} gradient and sequestering organelles, such as endoplasmic reticulum, vacuoles and mitochondria (Bok *et al.* 2001; Levina & Lew 2006; Bowman *et al.* 2009)? Many of the questions will require imaging of Ca^{2+} organellar pools in addition to cytoplasmic Ca^{2+}. Some may be revealed through the use of Ca^{2+} imaging using stable aequorin-expressing transformants (Nelson *et al.* 2004).

While many factors essential for polarity establishment and maintenance are known, our understanding of their interactions and precise molecular functions is still very limited. Key tasks for future research are to determine the identity and precise molecular function of the polarity markers. How are the different regulatory complexes such as the polarisome structurally and functionally interconnected? Do distinct polarisome subpopulations exist? How are the individual components recruited? What are their subcellular dynamics? How are polarisome and exocyst activities regulated? Are the different polarization events such as germination and subsequent hyphal extension as well as branch formation, controlled by identical or different regulatory networks? How are the Rho and Ras GTPase-GAP-GEF components organized into modules?

The Spitzenkörper and hyphal morphogenesis

The Spitzenkörper was first described as an iron-haematoxilyn stained body found at the apex of *Coprinus* spp. hyphae (Brunswik 1924). Light and electron microscopy analyses of different fungal species showed that a Spitzenkörper was present in all septate fungi including *Neurospora crassa* (Girbardt 1957; 1969; Grove & Bracker 1970). It is a pleomorphic and highly dynamic multi-component structure containing macrovesicles (apical vesicles), microvesicles, ribosomes and cytoskeletal components. The presence of the Spitzenkörper was correlated with the growing state of the hypha and also its growth directionality (Girbardt 1969; Bracker *et al.* 1997; Riquelme *et al.* 1998). By phase-contrast microscopy the *N. crassa* Spitzenkörper is characterized by having a dense phase-dark component surrounding, partly or fully, a phase-light smaller component (López-Franco & Bracker 1996; Roberson *et al.* 2010; Fig 1). Recent transmission electron microscopy and live imaging data have provided more detailed information on the organization and composition of the components that constitute the Spitzenkörper. The *N. crassa* Spitzenkörper is composed of a 'core' containing microvesicles (chitosomes), actin microfilaments, ribosomes and an uncharacterized amorphous material, and an outer accumulation of macrovesicles (Riquelme *et al.* 2002; Riquelme *et al.* 2007; Verdin *et al.* 2009; Delgado-Alvarez *et al.* 2010). In some instances (Fig 1C), the Spitzenkörper of *N. crassa* shows under phase contrast a phase-light region behind the main phase-dark body. It remains to be seen whether this morphology results from the pleomorphic behaviour of the Spk or it is a structure whose role and composition is unknown.

The presence of ribosomes in the Spitzenkörper core (Grove & Bracker 1970; Riquelme *et al.* 2002) indicates local protein synthesis at the hyphal tips. However, there are no studies indicating which proteins are synthesized at hyphal tips. Some potential candidates could be polarity markers, such as the polarisome component SPA-2 described above, which partially colocalized at the Spitzenkörper core in mature hyphae (Fig 1N).

Confocal microscopy of FM4-64 stained cells provides a practical fluorescent method to monitor the Spitzenkörper in living cells of *N. crassa* and other fungal species. Such staining by a dye used to monitor endocytosis, suggested an interconnection between exo and endocytosis (Fisher-Parton *et al.* 2000). Studies on the ontogeny of the Spitzenkörper revealed that in young germlings, an FM4-64 stained body was evident at the apex, before a Spitzenkörper could be observed by phase-contrast microscopy (Araujo-Palomares *et al.* 2007). Presumably, at early germination stages vesicles and other components of the Spitzenkörper had not reached a critical density and therefore could not be visualized by phase-contrast microscopy.

More recently, fluorescent tagging has assisted in the identification of some of the predicted components of the Spitzenkörper and discovered the presence of new components, providing clues as to the mode of operation of this structure during polarized growth. Four of the seven predicted chitin synthases in *N. crassa* localize in the core of the Spitzenkörper in mature hyphae using fluorescent-protein tags (Riquelme *et al.* 2007; Riquelme & Bartnicki-Garcia 2008; Sanchez-Leon *et al.* in press; Fajardo-Somera, unpubl.; Fig 1H,M), where microvesicles had been earlier seen by transmission electron microscopy (Fig 1B). In contrast, GS-1, a protein needed for glucan synthase activity, was found in the most outer layer of the Spitzenkörper (Verdin *et al.* 2009; Fig 1M), where mainly macrovesicles were observed by transmission electron microscopy (Grove & Bracker 1970; Riquelme *et al.* 2002; Fig 1A). These findings, besides corroborating the spatial stratification of the Spitzenkörper identified in earlier studies, show that there is an associated functional stratification, and demonstrate that the Spitzenkörper is part of the apparatus that builds the hyphal cell wall.

The presence of F-actin in the Spitzenkörper core, first detected by immunolabeling in *Magnaporthe grisea* (Bourett &

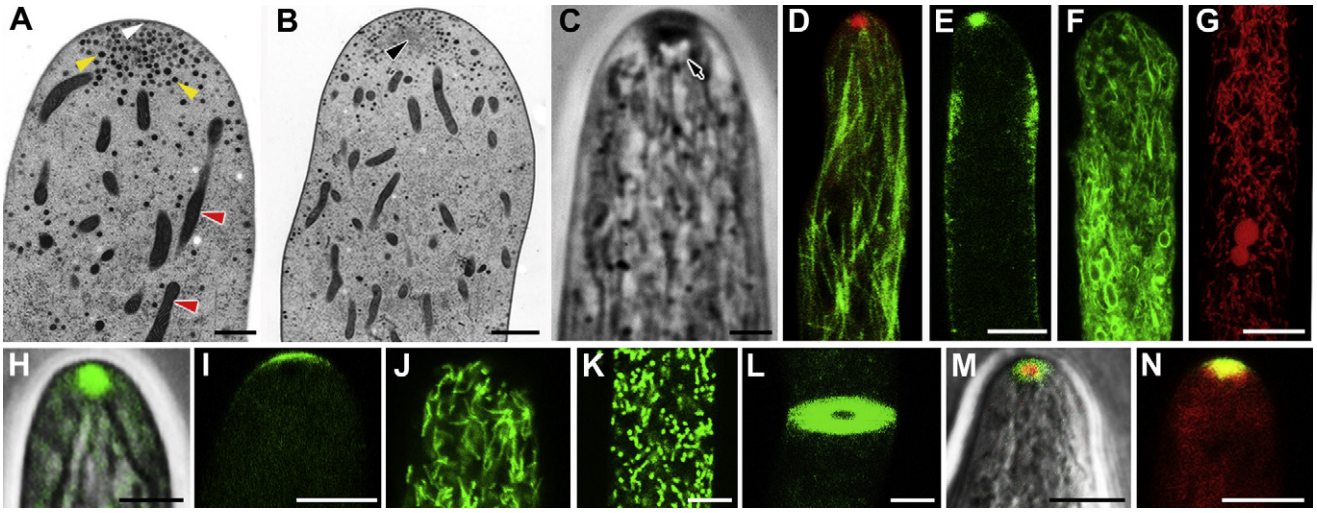

Fig 1 – Structure and ultrastructure of cellular components in *N. crassa* hyphae. (A) TEM of a hyphal tip showing accumulation of macrovesicles (yellow arrowheads), some of them fusing with the cell surface (white arrowheads), mitochondria (red arrowheads), and some microtubules. Scale bar 0.8 μm. (B) TEM of a medial section of a hyphal tip showing the accumulation of microvesicles at the Spitzenkörper core (black arrowhead). Scale bar 1.4 μm. (C) Phase-contrast microscopy showing the phase-dark Spitzenkörper and a phase-light body near the back of the Spitzenkörper (black arrow). Scale bar 1.7 μm. (D) Laser scanning confocal microscopy (LSCM) of a heterokaryon showing microtubules labelled with GFP and CHS-1 labelled with mChFP. (E) LSCM showing Lifeact-GFP at the Spitzenkörper core and the cortical subapex (Delgado-Alvarez *et al.* 2010). Scale bars for D, E, 5 μm. (F) ER at the hyphal tip of a strain expressing GFP-tagged NCA-1, a protein encoding a CA-transporting ATPase. (G) Vacuoles in the region approximately 300 μm behind the apical tip, as visualized by fusing RFP to CAX, a calcium-H^+ exchange protein. Scale bars for F, G, 10 μm (H) Overlap of phase-contrast and LSCM showing CHS-1-GFP at the Spitzenkörper core. (I) Exocyst component SEC-6 tagged with GFP by LSCM. (J–K) ARG-4, a mitochondrial enzyme of the arginine metabolic pathway, fused to GFP shows how mitochondria exhibit different structures at the apical tip region (J), and at regions approximately 1mm distal to the tip (K). (L) 3D reconstruction of a completed septum in a strain expressing CHS-1-GFP. (M) Heterokaryon showing GS-1-GFP at the Spitzenring and CHS-1-mChFP at the Spitzenkörper core. (N) Heterokaryon showing the polarisome component SPA-2 tagged with GFP and CHS-1-mChFP. Scale bars for H–N, 5 μm.

Howard 1991), was confirmed in *N. crassa by* immunolocalization studies (Virag & Griffiths 2004) and more recently by live cell imaging with Lifeact (Berepiki *et al.* 2010; Delgado-Alvarez *et al.* 2010; Fig 1E).

The Spitzenkörper is believed to function as a vesicle supply center (VSC) that regulates the delivery of cell wall-building vesicles to the apical cell surface (Bartnicki-Garcia *et al.* 1989). By programming a VSC to advance as the Spitzenkörper in video-microscopy recorded sequences of *N. crassa* growing hyphae, while at the same time distributing 'cell growing units' (equivalents of vesicles) towards the cell surface, it was possible to mimic, by computer simulation, the hyphal morphogenesis of *N. crassa* wild-type and mutant strains (Riquelme *et al.* 1998; 2000).

Collectively, the gathered evidence shows that the Spitzenkörper presumably behaves as a very sophisticated exocytic apparatus, maintaining a delicate functional and structural balance of the different types of vesicles dedicated to make cell wall. The suspected tethering of vesicles to the plasma membrane is controlled by the exocyst, an octameric protein complex conserved from yeast to mammalian cells (Terbush *et al.* 1996; He & Guo 2009). The eight components are SEC-3, SEC-5, SEC-6, SEC-8, SEC-10, SEC-15, EXO-70, and EXO-84. Even though in *N. crassa* all exocyst components tagged with GFP accumulate primarily as a crescent adjacent to the cell surface at the hyphal dome (M.R. & M.F, unpubl.; Fig 1I), where presumably intensive exocytosis occurs, so far no direct study on the function of exocyst components in *N. crassa* has been reported. However in a screen for temperature-sensitive polarity mutants, strains affected in *sec*-5 were isolated (Seiler & Plamann 2003). Under restrictive growth conditions the mutant forms compact colonies consisting of bulbous hyphae, suggesting apolar fusion of secretory vesicles with the plasma membrane.

Studies in *Saccharomyces cerevisiae* suggest that Sec3p and Exo70p are recruited to growth sites in an actin independent manner (Boyd *et al.* 2004). Proper localization of both components depends on their interaction with the phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP_2) (He *et al.* 2007; Liu *et al.* 2007; Zhang *et al.* 2008), and Sec3p recruitment requires interaction with the Rho-type GTPase Rho1p (Guo *et al.* 2001). The remaining exocyst components and additional Exo70p are thought to be transported to growth sites *via* secretory vesicles (Boyd *et al.* 2004). At the plasma membrane the exocyst complex then assembles resulting in vesicle tethering. Recently however, this view has been challenged by an elegant study by Jones & Sudbery (2010) analyzing the dynamics of polarisome, exocyst and Spitzenkörper components in

Candida albicans. While Spitzenkörper components were highly dynamic, polarisome components remained more stable at the cell tips. Exocyst factors showed intermediate behaviour suggesting that they belong to the more stable residing cell surface factors.

Many questions concerning the structure, function, activity and dynamics of the highly conserved exocyst complex remain unanswered. Because of its large hyphal size and fast growth *N. crassa* provides an ideal model for further investigation of these topics.

There is a clear need to elucidate the composition of of the different types of vesicles that accumulate at the Spitzenkörper and to determine how these different vesicles fuse with the plasma membrane to either release their content to the extracellular matrix or to provide transmembrane proteins. Another unresolved question is whether all vesicles reaching the apex accumulate at the Spitzenkörper. One possible mechanism for maintaining an appropriate volume and constitution of vesicles associated with the Spitzenkörper would be the redirection of excess vesicles to distal hyphal areas, as predicted by the VSC model. The commonly observed retrograde transport of vesicles observed in hyphae supports this possibility.

Cell architecture

The cytoskeleton

The fungal cytoskeleton is a dynamic structure that maintains shape, organization and support of cytoplasmic components, control of cell movements, and plays important roles in both intracellular transport of vesicles and organelles, and cellular division. The fungal cytoskeleton is composed primarily of two protein filaments, the microtubules (MTs) and the actin microfilaments (MFs). Each cytoskeletal element has distinct mechanical and dynamic characteristics and performs specific, as well as, shared duties in the cell. Their function and behaviour are direct results of the inherent characteristics of their proteins as well as the activities of interacting proteins and cytoplasmic components in highly regulated and precise ways.

Microtubules

The *Neurospora crassa* microtubular cytoskeleton is clearly more complex than that of other filamentous fungi (e. g. *Aspergillus nidulans* and *Ustilago maydis*). MTs are tubular structures built from subunits of α- and β-tubulin heterodimers, that assemble end to end, forming 13 parallel protofilaments that bundle together to build the wall of the MT. MTs have an outer diameter of 24 nm and a variable length. The lumen of the MTs has a diameter of 14 nm and is routinely described as empty. However, there is clear evidence that dense particles and fibrous materials reside within the core of the MTs, which may represent MT-binding proteins that regulate their assembly and disassembly (Garvalov *et al.* 2006).

In *N. crassa*, MTs labelled with β-tubulin-GFP occupy both the cortical and central cytoplasmic hyphal regions. However, in apical hyphal compartments they are preferentially concentrated in the central cytoplasm and they are long and longitudinally arranged along the hypha (Mouriño-Pérez *et al.* 2006). Straight MTs are rarely seen in either parent or branch hyphae. Most MTs exhibit a slight, yet distinct helical curvature with a long pitch and a tendency to intertwine with one another to form a loosely braided network throughout the cytoplasm (Fig 1). Oblique or transverse MT orientations are observed during branch formation and in association with the mitotic spindle. Cytoplasmic MTs are mostly solitary, although bundles of two to four sometimes occur. Microtubules extend into the apical dome and often transverse the Spitzenkörper. Other MTs terminate at the periphery of the Spitzenkörper or the apical plasma membrane. As hyphae elongate, there is continuous rotation of the MT network along the hyphal axis suggesting that the MTs advance and rotate as a component of the cytoplasmic bulk flow (Mouriño-Pérez *et al.* 2006). Anti-actin drugs such as cytochalasin A have a strong effect on the organization and orientation of MTs (Ramos-García *et al.* 2009).

In subapical compartments, MTs are less longitudinally orientated than at the apex and further back they become randomly arranged. MTs in apical hyphal compartments appear longer than those further back from the colony periphery. MTs extend through septal pores and are forced into closer proximity with each other as they transverse the pore (Freitag *et al.* 2004). Apparent bundles of MTs are observed in spindles of nuclei undergoing mitosis, and these structures are most obvious in subapical hyphal compartments (Freitag *et al.* 2004). Mitotic spindles appear randomly oriented and positioned within hyphae. Astral microtubules extend from each end of the spindles and sometimes appear to connect with the plasma membrane.

As in other eukaryotic cells, MTs display dynamic instability; they are extremely dynamic and exhibit growth and shrinkage due to the rapid interconversion of assembly and disassembly at the MT plus-ends. The dynamic nature of MTs allows the formation of different structural organizations during cell cycle, growth, and development. In *N. crassa* this is best observed in germ tubes, where MTs are narrower and less abundant than in mature leading hyphae (Uchida *et al.* 2008). Fragmentation of MTs and their subsequent anterograde and retrograde movements have been reported in the hyphal cortex of *N. crassa* (Uchida *et al.* 2008). These actions suggest the presence of MT-severing proteins (e.g., katanin) and treadmilling or active MT transport.

In *Ustilago maydis* and in *A. nidulans*, the MTs plus-ends are directed to the hyphal tip (Zhang *et al.* 2003; Konzack *et al.* 2005; Schuchardt *et al.* 2005), whereas in *N. crassa* there is a mixed polarity of MTs at the tip. Fluorescence Recovery after photobleaching (FRAP) experiments showed evidence of nucleation and retrograde polymerization of MTs at the tip, in close proximity to the plasma membrane (Mouriño-Pérez *et al.* 2006).

Mutants defective in microtubule-associated proteins and molecular motors have improved our understanding on the mechanistic aspects of intracellular motility in *N. crassa* hyphae (Plamann *et al.* 1994; Minke *et al.* 1999b; Tinsley *et al.* 1998; Kirchner *et al.* 1999; Seiler *et al.* 1997, 1999, 2000; Steinberg & Schliwa 1995; Riquelme *et al.* 2000, 2002; Seiler & Plamann 2003). *Neurospora crassa ro-1* hyphae, defective in cytoplasmic dynein, showed vesicles, mitochondria, and nuclei

altered to varying degrees, an erratic and reduced Spitzenkörper, disrupted MTs distribution and distorted hyphal morphogenesis (Riquelme *et al.* 2000, 2002; Ramos-García *et al.* 2009). Whereas *N. crassa nkin* hyphae, which lack conventional kinesin (*nkin-1*), failed to establish a Spitzenkörper, showed abnormal mitochondrial positioning, had slight deffects on MTs organization and on nuclear shape (Seiler *et al.* 1997, 1999; Ramos-García *et al.* 2009; R.M-P., unpubl. results). Additionally, *ro-1* and *nkin-1* are involved in regulating microtubule dynamic instability in mature hyphae, but not in germ tubes. Though it is unclear what specific roles microtubule motors play, it seems likely that together with microtubule plus-end associated proteins (+TIPS) contribute to microtubule dynamics and, consequently, hyphal growth (Uchida *et al.* 2008).

One of the intriguing questions that remain unanswered is the role of MTs in hyphal growth. MTs presumably support hyphal extension (Fuchs *et al.* 2002; Horio & Oakley 2005). However when they are depolymerized, *N. crassa* hyphal extension continues, albeit with a marked loss of growth directionality. This suggests MTs are not needed for transport of material needed for cell growth to the hyphal tips, but are necessary to stabilize the Spitzenkörper and maintain hyphal morphogenesis. Although some efforts have been directed to study the localization and trafficking of MAPs and microtubule-associated motor proteins (R.M-P., unpubl.; M.P., unpubl), no studies are available in *N. crassa* showing the cargos transported along MTs.

Actin

Actin microfilaments (MFs) are composed of subunits of identical actin monomers that assemble into two protofilaments, forming a left-handed helical filament about 7 nm in diameter. These short and flexible filaments are generally present in much higher numbers in the cytoplasm than MTs.

In recent years, many studies have reaffirmed the central importance of F-actin and associated proteins in growth and spatial regulation of organelles in tip-growing cells (Harris & Momany 2004; Virag & Griffiths 2004; Harris *et al.* 2005). In *Neurospora crassa*, initial studies used various methods such as anti-actin antibodies to label actin in fixed cells. Filamentous actin is notoriously difficult to preserve during fixation. Nevertheless, using immunolabeling, actin has been previously observed in the Spitzenkörper of *N. crassa* (Heath *et al.* 2000; Virag & Griffiths 2004; Harris *et al.* 2005). The population of F-actin in the Spitzenkörper has been proposed to regulate vesicle delivery and/or fusion at the growth site (exocytosis), and may also regulate calcium channels, whose activity is important for tip growth (Harris *et al.* 2005).

Recently, live cell imaging of F-actin has been carried out in *N. crassa* using green fluorescent protein (GFP) fused to G-actin and to different F-actin binding proteins (ABPs) such as fimbrin, tropomyosin, and Lifeact, an actin marker consisting of the first 17 residues of yeast Abp140p (Berepiki *et al.* 2010; Delgado-Alvarez *et al.* 2010). The studies done in living cells showed that although actin is found throughout the cell, the highest density of actin filaments is at the cell cortex. The cortex is also the site for most MF nucleation. Like MTs, actin MFs are polar structures and are regulated through the interactions of many associated proteins. Imaging of the actin cytoskeleton, including actin associated proteins, reveals several distinct arrangements and distribution patterns in *N. crassa*. These include small spots or patches, longitudinal cables, and contractile rings associated with septum formation. Small cortical patches are typically concentrated in a band located between 1 and 4 μm behind the growing tip of a mature hypha (Fig 1E; Delgado-Alvarez *et al.* 2010). Actin patches are excluded from the extreme hyphal tip and are generally present in reduced numbers in the lower subapical hyphal areas. It has been shown that proteins as Arp2/3 complex, coronin and fimbrin colocalize with actin cortical patches in mature hyphae of *N. crassa* (Delgado-Alvarez *et al.* 2010; Echauri-Espinosa, unpubl.), supporting a spatially coupled mechanism of apical exocytosis and subapical endocytosis *via* actin patches. In addition to cortical patches, a small apical aggregation or spot of actin label with the chimeric protein Lifeact has been reported. This aggregation is at the core of the Spitzenkörper (Fig 1E; Delgado-Alvarez *et al.* 2010).

It has been suggested that the Spitzenkörper is a 'switching station' where vesicles are transferred from MT tracks to actin tracks. The convergence of cytoplasmic Mts onto the Spitzenkörper and the presence of actin inside the Spitzenkörper seem to lend support this idea but experimental evidence is obviously needed to demonstrate such transfer.

The structure and distribution of organelles in fungal hyphae

With the notable exception of plastids, fungal hyphae contain the full complement of organelles found in other types of eukaryotic cells. However, the structure and distribution of organelles is not uniform in all parts of the hypha. Rigorous examination by transmission electron microscopy has shown that the typical fungal hypha can be characterized as having at least four different regions (see Roberson *et al.* 2010 for a recent review). The first 1–5 μm of the hyphal tip contains the Spitzenkörper, some mitochondria, and occasionally smooth endoplasmic reticulum (ER) and Woronin bodies (WBs). Behind that region is an area 2–4 times as large that contains mitochondria and some ER cisternae but lacks most of the other major organelles, including nuclei. The third region, which extends to the first septum, contains the complete collection of organelles. Distal to the septum are older hyphal segments, which also contain all the organelles, but their structure and abundance is often different from what is observed in the apical and subapical regions. Transitions from long germ tubes with immature phase-grey Spitzenkörper to mature hyphae with a phase-dark and more sharply delimited spherical Spitzenkörper are accompanied by a reorganization of most organelles which were uniformly distributed in the germ tube into different zones of the hypha (Araujo-Palomares *et al.* 2007).

In recent years living hyphae of *Neurospora crassa* have been examined by labelling them with fluorescent dyes or by fluorescently tagging their specific proteins (for examples see Bowman *et al.* 2009; Freitag *et al.* 2004; Hickey *et al.* 2004). The results from these types of experiments complement the observations made with the transmission electron microscope, with each approach having its own advantages and limitations. It is also important to remember that the external and internal structure of hyphae may vary with different growth regimes e.g., submerged in liquid, on the surface of agar or in

the air. Almost all of the published observations with live cells have been made with hyphae growing on an agar surface.

Mitochondria are the most abundant and most uniformly distributed organelles in hyphae. Almost all regions, from just behind the Spitzenkörper to and including the older hyphal segments contain numerous mitochondria. Their size and shape, however, does vary with position. In *N. crassa*, for example, mitochondria in the apical segment are long thin tubes (~0.3 μm wide ×5–10 μm long) generally aligned along the long axis and reaching at times the posterior zone of the Spitzenkörper (Fig 1J). In segments behind the first septum the mitochondria are much shorter (2–3 μm) and randomly oriented (Fig 1K). The molecular processes that control their changes in size and distribution are not understood.

Neurospora crassa colonies are comprised of multinucleate hyphae, forming syncytia in which each compartment can easily comprise dozens of nuclei. Even though the nuclei are relatively large (3–4 μm) they move readily through septal pores and are distributed quite uniformly through all regions of the hyphae, except for an exclusion zone that extends ~50 μm from the tip (Freitag *et al.* 2004; Ramos-García *et al.* 2009). In the apical compartment nuclei also appear more variable in size, with a significant number of nuclei that are much smaller than those observed in older hyphal segments (Bowman *et al.* 2009). In wild-type hyphae, nuclei are usually elongated (oval or pear-shaped), but in strains with mutations in motor proteins nuclei tend to be spherical. Those nuclei in mitosis are immobile while the others generally move towards the apex. Cytoplasmic flow is the major motive force, but motors proteins are likely to be involved in the retrograde and rapid anteriograde movement that is also observed (Freitag *et al.* 2004, Ramos-García *et al.* 2009).

As in other organisms the nuclear envelope is a dual membrane that gives rise to the endoplasmic reticulum (ER) (Bowman *et al.* 2009). GFP- and RFP-tagged proteins predicted to be in the endoplasmic reticulum are enriched in the rough ER (RER) around the nuclear envelope and around poorly resolved membranes, likely to be smooth ER (Fig 1F). In electron micrographs ER cisternae are very thin, less than 0.05 μm, and scattered throughout the cytosol, which may explain why they are relatively indistinct when viewed with GFP or RFP. In the apical segment the membraneous component of the ER is abundant and these membranes are observed within a few μm of the apex. In older segments most of the tagged ER maker proteins are associated with the nuclear envelope.

The Golgi apparatus in fungi is not a discrete organelle. Recent work with *Saccharomyces cerevisiae* (Losev *et al.* 2006), *Aspergillus nidulans* (Pantazopoulou & Penalva 2009), and *N. crassa* (Bowman *et al.* 2009) shows that different Golgi-localized proteins are often in different, non-overlapping vesicular compartments. In *A. nidulans* some of these compartments have been visualized as tubular or ring structures (Pantazopoulou & Penalva 2009). Organelles that form tubular rings and protrusions were previously observed in electron micrographs and were assumed to be Golgi cisternae (Roberson *et al.* 2010). Golgi equivalents are more abundant in the subapical region of the hyphal tip than in older regions.

The organelle with the most variable structure is the vacuole (Bowman *et al.* 2009; Cole *et al.* 1998; Fisher-Parton *et al.* 2000). Electron micrographs showed spherical and tubular compartments with a wide range of sizes, but it was difficult to know if these compartments were indeed functionally the same. GFP- and RFP-tagged vacuolar proteins (e.g., VAM-3, VMA-1, CAX), are also seen in membrane compartments of variable size and structural diversity, which supports the idea that all these organelles are types of vacuoles (Fig 1G). The region of the hypha near the apex is largely devoid of vacuoles. Further back, but before the first septum, the vacuolar markers are localized in a network of interconnected tubules. Distal to the first septum the tubular network disappears, and spherical vacuoles in a wide range of sizes predominate. Hyphae that are injured or stop growing can become filled with large spherical vacuoles. In filamentous fungi we know almost nothing about what determines the structure and abundance of vacuoles.

WBs are peroxisome-related membrane-bound organelles slightly larger than the septal pore and found at the cell periphery or in association with the septum (Markham & Collinge 1987). They seal the septal pore in response to cellular wounding in Ascomycetes (Collinge & Trinci 1974; Markham & Collinge 1987). In *N. crassa*, HEX-1 was identified as the crystalline subunit of the matrix of the WBs (Jedd & Chua 2000). In a forward genetic screen to isolate *N. crassa* mutants defective in WB biogenesis, a Woronin sorting complex (WSC) was identified at the membrane of large peroxisomes, where it self-assembles into detergent-resistant oligomers that envelop HEX-1 protein assemblies and produce nascent WBS (Liu *et al.* 2008). More recently, a *N. crassa* Leashin tether has been analyzed (Ng *et al.* 2009), which promotes WB inheritance and holds the organelle in position (*via* WSC) until signals from cellular damage induce release, translocation to the septal pore and membrane resealing. In contrast to most fungal species, where WBs are tethered directly to the pore rim, in *N. crassa* they have evolved a delocalized pattern of cortex association, based on the unique two-gene structure of the *lah* locus. The locus is comprised of genes encoding LAH-1, which links WBS with the cell cortex and not the septal pore, and LAH-2 which localizes to the hyphal apex and the septal pore rim and plays a role in colony development. This two-gene structure may also play a role in the rapid hyphal growth capability of *N. crassa* as the tethering of WBS to the cell cortex (and keeping septal pores clear) minimizes restrictions on cytoplasmic streaming, which is a likely prerequsite for rapid growth rates.

As more and more fluorescent confocal images accumulate tracing the localization of specific proteins in the fungal cell, their precise relationship to the organelles or structures revealed by transmission electron microscopy is not always clear. There is in general an urgent need to reconcile the images obtained by fluorescence microscopy with the images obtained by transmission electron microscopy so that the location of a fluorescent-labelled protein could be assigned unambiguously to the corresponding subcellular structure. One helpful advance would be a high-resolution 3D mapping of the internal organization of a hypha, i.e., an updated and extended version of the classic reconstructurion made by Girbardt (1969). The new techniques of electron tomography

promise to be of great help in achieving these goals (McIntosh *et al.* 2005).

The cell wall: structure and functions

The cell wall is a structure common to all fungi. It plays a key role in defining the morphology of the fungal cells. It provides protection from environmental stresses, varying its composition in response to a changing environment. Some of the cell wall proteins have been shown to be upstream elements of signal transduction pathways regulating fungal growth, morphology and development. The wall is a dynamic and malleable structure, which presumably undergoes remodelling to accommodate hyphal branching, cell fusion events, and developmental processes. Despite the importance of the cell wall, a rather limited amount of information is available concerning its structure and biosynthesis.

Studies from the 1960's demonstrated the presence of glucan and chitin in the *Neurospora crassa* cell wall, and that the wall contained glucose, glucosamine, mannose, galactose and galactosamine (Bartnicki-Garcia 1968; De Terra & Tatum 1963; Mahadevan & Tatum 1965). A recent analysis of the cell wall shows the presence of glucose, N-acetylglucosamine, mannose and galactose and a glucosyl linkage analysis showed the presence of large amounts of 1,3 linked glucose (Bowman *et al.* 2006; Maddi *et al.* 2009). Studies have demonstrated the presence of β-1,3-glucan and the importance of β-1,3-glucan synthase in cell wall biosynthesis (Taft & Selitrennikoff 1988; Tentler *et al.* 1997). α-1,3-glucans have been found in other fungal cell walls and the *N. crassa* genome encodes two α-1,3-glucan synthase genes (NCU02478 and NCU08132), so some of the 1,3 linked glucose could be found as an α-1,3-glucan. While the yeast cell wall contains a large amount of β-1,6-glucan, which serves to cross-link the other constituents together, linkage analyses show that cell walls of *Aspergillus fumigatus* and *N. crassa* are devoid of β-1,6-glucan (Fontaine *et al.* 2000). An analysis of the *N. crassa* genome shows that homologues of the *Saccharomyces cerevisiae* enzymes responsible for the synthesis of β-1,6-glucan are lacking (Borkovich *et al.* 2004). *Neurospora crassa* has been utilized to advance fungal cell wall research in many ways, including the description of chitosomes and subsequent localization of chitin synthase in vesicular organelles (Bartnicki-Garcia *et al.* 1978; Sietsma *et al.* 1996), the cloning of a first chitin synthase from a filamentous fungus (Yarden & Yanofsky 1991) and the use of partial chitin synthase gene sequences as a phylogenetic tool (Carbone & Kohn 1999).

Cell wall components, including chitin, glucan, and glycoprotein, are delivered to the cell wall space and then subsequently cross-linked together to form a cell wall matrix. A model of the proposed structure for the *N. crassa* cell wall is provided in Fig 2. There are seven chitin synthase genes within the *N. crassa* genome (Riquelme & Bartnicki-Garcia 2008). Four of the seven chitin synthases (CHS-1, -3, -5, and -6) and a regulatory subunit of the glucan synthase complex (GS-1) are delivered from sites of vesicle formation to the Spitzenkörper and from there to the plasma membrane near the tip of *N. crassa* growing hyphae in two clearly distinct vesicle populations that as shown above are located in different layers of the Spitzenkörper (Riquelme *et al.* 2007; Verdin *et al.* 2009; Sanchez-Leon *et al.* in press). The Spitzenkörper functions as a vesicle-sorting center during the delivery of the chitin synthases and glucan synthase to the plasma membrane. The substrates for glucan and chitin synthesis, UDP-glucose and UDP-N-acetyl-glucosamine are present in the cytosol and probably delivered directly without vesicular transport (Martinez *et al.* 1987; Taft & Selintrennikoff 1988). The newly synthesized chitin and β-1,3-glucan are extruded into the cell wall space during synthesis. In addition to the glucans and chitin, *N. crassa* cell walls have been shown to have galactomannan-containing glycoproteins. The cell wall proteins are synthesized on ER-associated ribosomes and pass through the classical ER to Golgi secretory pathway on their way to the cell wall. The galactomannan is synthesized as O-linked and N-linked post-translational modifications on cell wall proteins as the protein passes through the ER and Golgi apparatus. Characterization of the galactomannans associated with these cell wall proteins has given a proposed structure consisting of a short core chain of α-1,6-mannose residues with short α-1,2-mannose-containing side chains capped by β-linked galactofuranose residues (Nakajima *et al.* 1984). Between 80 % and 90 % of the mass of the cell wall is found in the glucan and chitin polymers while glycoproteins account for the remaining 10 %–20 % of the cell wall.

Cell wall proteins include those cross-linked into the glucan/chitin matrix and those that are not covalently attached to the wall but are tightly associated with it. Proteomic experiments have revealed the presence of 26 major proteins that are cross-linked into the glucan/chitin matrix in *N. crassa* (Maddi *et al.* 2009). These proteins included 'structural proteins' which lack any known enzymatic activity and a number of glycosylhydrolases and glucosyltransferases, which are presumed to function in cross-linking the wall polymers and proteins together. All of the *N. crassa* major integral cell wall proteins have close homologues in the sequenced genomes from other fungi and yeast (Maddi *et al.* 2009), suggesting that these proteins have been evolutionarily conserved. They most certainly play important roles. Yet, knockout mutations in the genes encoding most of these proteins did not result in major changes in morphology (Free, personal observation). This may be due to a large amount of 'functional redundancy' between the different cell wall proteins. Alternatively, it may reflect the fact that the fungi have a 'cell wall stress response' (Klis *et al.* 2006), which is activated when the wall is under stress and directs the synthesis of additional cell wall proteins that could be compensating for the missing protein in the mutants.

Over half of the major *N. crassa* cell wall proteins are synthesized as GPI-anchored proteins (Maddi *et al.* 2009). As the cell wall proteins pass through the secretory pathway, they are extensively modified by the addition of GPI-anchors and O-linked galactomannans, and by the addition of galactomannan to N-linked oligosaccharides. These post-translational modifications are critical for the formation of the hyphal cell wall. Cells with mutations affecting the biosynthesis of the GPI anchor grow in a tight colonial manner and are characterized by having weakened cell walls (Bowman *et al.* 2006). Similarly, *mnt-1* (NCU01388) mutants, which are affected in an α-1,2-mannosyltransferase that functions in the addition

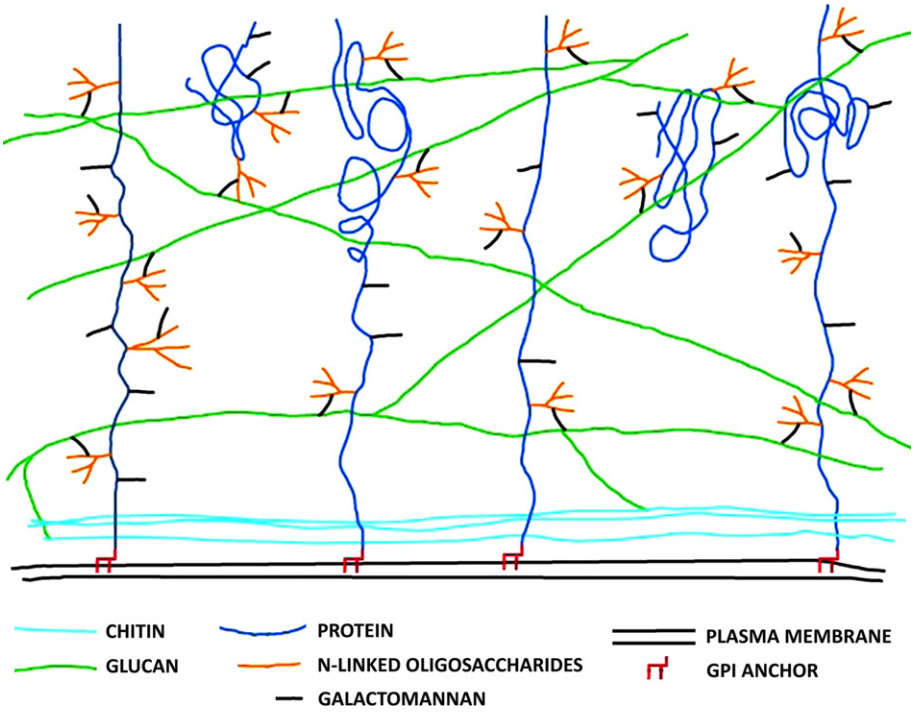

Fig 2 – A schematic representation of the *N. crassa* cell wall structure. Polysaccharides (glucans, chitin and galactomannans) constitute the 80–85 % composition of the cell wall. Glycoproteins consitute the remaining 15–20 %.

of O-linked oligosaccharides, and *och-1* (NCU00609) mutants, which are affected in an α-1,6-mannosyltransferase that functions in the addition of a galactomannan to N-linked oligosaccharides, are unable to generate a normal cell wall, and grow in a tight colonial mode (Bowman *et al.* 2005; Maddi & Free 2010).

One of the most pressing questions concerning the formation of the cell wall is how and when the chitins, glucans, and glycoproteins become cross-linked together and how that process is regulated to allow the cell wall to be a dynamic structure. The glycosylhydrolases and glycosyltransferases in the cell wall are thought to be responsible for doing the cross-linking, but a great deal remains to be learnt about the pecificity of these enzymes and how they accomplish the cross-linking of the cell wall components. The *S. cerevisiae* gas1p and the *A. fumigatus* GEL1 proteins have been shown to function as glucanhydrolases/glucantransferases capable of lengthening and shortening β-1,3-glucan polymers, and mutants affected in these enzymes have cell wall defects (Mouyna *et al.* 2000). *Neurospora crassa* has five GEL1 homologues, and mutants in two of these have been found to have cell wall defects (Free, unpubl.). The *S. cerevisiae* Crh1p and Crh2p cell wall proteins cross-link β-1,6-glucan and chitin polymers (Cabib *et al.* 2006), and the *N. crassa* homologue, GH7-16 (NCU05974) is found among the major cell wall proteins cross-linked into the glucan/chitin matrix (Maddi & Free 2010). The *N. crassa och-1* mutant is unable to cross-link cell wall proteins into the glucan/chitin matrix demonstrating that the cross-linking of protein into the wall requires the presence of some elements of the galactomannan found on modified N-linked oligosaccharides, and suggests that the cross-linking may be occurring between N-linked oligosaccharides and elements of the cell wall glucan/chitin matrix. Although we have some basic information about the carbohydrate and protein composition of the *N. crassa* cell wall, it is clear that there is much left to be learnt. For example, we would like to know how the cell wall is generated at the tip of the growing hyphae. We need to learn more about how and where the cross-linking of cell wall components occurs. The important questions of how the cell wall is remodelled to accommodate branch formation and how the cell wall composition is changed to generate morphologically different tissues remain to be elucidated.

Development

The formation and regulation of the septum – separating between cells, yet maintaining cytoplasmic continuity

Cytokinesis is tightly regulated to ensure that each daughter cell receives the correct complement of DNA and other cellular constituents. Cell division can be divided into three general steps that apply to most eukaryotic cells (Barr & Gruneberg 2007): the selection of the future division plane, the assembly of a cortical acto-myosin ring (CAR) at this site, and its constriction coupled with membrane invagination. In fungi, there is the additional formation of a cross wall, the septum, composed of glucans, chitin and other extracellular polysaccharides. After its coverage by additional layers of cell wall material that form two secondary septa, the primary septum is dissolved by hydrolytic enzymes to allow cell separation in the unicellular yeasts or conidiospore formation during asexual development of filament-forming species. The septation machinery is finally removed from the septum.

Nuclear behaviour and cortical landmark proteins may specify septum placement

In contrast to unicellular fungi, not every nuclear division is coupled with cytokinesis in filament-forming fungal species, resulting in the formation of multinuclear hyphal compartments. Thus nuclear position and cell cycle seems only loosely coordinated with septum placement. Nevertheless, CAR assembly and septum formation is clearly controlled through nuclear position and cell cycle progression in *Aspergillus nidulans* (Harris *et al.* 1994; Wolkow *et al.* 1996; Momany & Hamer 1997). This may potentially also apply to *Neurospora crassa* but the connection between nuclear cycle and septum positioning is blurred by *N. crassa*'s asynchronous nuclear divisions (Serna & Stadler 1978; Minke *et al.* 1999a, Plamann *et al.* 1994). Interestingly, anucleate tip cells and internal compartments are frequently observed in *N. crassa ropy* mutants that are defective in nuclear distribution (Plamann *et al.* 1994), but multiple neighbouring anucleate compartments are almost never detected (Minke *et al.* 1999a). Thus a mechanistic connection between nuclear position, nuclear cycle and septum placement may also exist in *N. crassa*, but is difficult to detect. After obtaining the relevant mutants or producing the appropriate strains, *N. crassa* may provide an excellent model to dissect the molecular basis of asynchronous cell cycles within a common cytoplasm.

More direct evidence for a connection between the nuclear cycle and septation provides the analysis of components of the septation initiation network (SIN, also called mitotic exit network - MEN), which is a critical signalling cascade that connects cell cycle progression with the initiation of cytokinesis in budding and fission yeast (McCollum & Gould 2001; Krapp & Simanis 2008). Recent studies confirm the presence of most components of the SIN in *N. crassa* (Dvash *et al.* 2010; März *et al.* 2009; Seiler & Justa-Schuch 2010). Deletion of the *dbf-2* gene, encoding the final kinase of the SIN cascade, and of its co-activator *mob-1* results in aseptate strains that are unable to produce macroconidia. Moreover, elongated nuclei are detected in vegetative hyphae, and abnormal meiotic progeny is observed in the two mutants, supporting weak cell cycle defects, but not a complete block in mitosis (März *et al.* 2009; Dvash *et al.* 2010). This is reminiscent of the situation observed in *Schizosaccharomyces pombe*, where mutations in positive SIN components lead to growth arrest after multiple rounds of mitosis in non-dividing cells (Krapp & Simanis 2008).

The mechanism for determining the site of cell division is one of the least-conserved aspects of cytokinesis in eukaryotic cells. Budding and fission yeast, for example, have developed fundamentally distinct mechanisms to ensure proper nuclear segregation. The bud site selection system of *Saccharomyces cerevisiae* uses cortical cues from the previous cell division cycle, while opposing nuclear and cell end-dependent spatial signals are integrated by the *S. pombe* specific landmark protein Mid1 (Chang & Peter 2003). Nevertheless, in both cases the anillin-type scaffolds and Bud4p and Mid1, respectively, are critical for temporal-spatial organization of division site selection (Park & Bi 2007; Martin 2009). The *N. crassa* homologues of the *S. cerevisiae* axial bud site marker proteins Bud3p and Bud4p are essential for septum formation. Both proteins appear prior to the formation of a detectable septum as cortical rings at incipient septation sites that contract with the forming septum (Justa-Schuch *et al.* 2010). Moreover, *N. crassa* BUD-4 appears first as motile cortical dots in internal regions of the hypha that subsequently coalesce into cortical rings, suggesting a function of BUD-4 in specifying future septation sites. However, it is currently unknown, if BUD-4 determines the placement of the future septation site or if it marks a previously selected site (e.g., by the SIN).

Assembly and function of the CAR machinery

S. pombe is currently the best-studied model for the assembly of the CAR, which occurs by the ordered recruitment of ring components to cortical nodes at the cell center and their maturation into the contractile ring (Pollard & Wu 2010). Of the many (>100) proteins that are required for CAR assembly and function (i.e., IQGAP, formin, F-BAR domain proteins, type II myosin, and distinct Rho GTPases), only the formin BNI-1, the anillin, and the RHO-4 GTPase module have been characterized and shown to localize to forming septa in *Neurospora crassa* (Rasmussen & Glass 2007; Justa-Schuch *et al.* 2010; Seiler & Justa-Schuch 2010). Genetic and biochemical evidence identify BUD-3 as a guanine exchange factor (GEF) for the Rho GTPase RHO-4, which is also essential for septum formation and functions upstream of CAR assembly (Justa-Schuch *et al.* 2010; Rasmussen & Glass 2005, 2007). The anillin-like scaffold BUD-4 acts as landmark to initiate septation by recruiting the BUD-3-RHO-4 module to the cortex. The recruitment of the formin BNI-1 to the site of CAR assembly is abolished in *bud-3, bud-4* and *rho-4* mutants indicating that formin localization depends on BUD-3, BUD-4 and RHO-4. The localization of BUD-3 as a cortical ring prior to septum initiation depends on the presence of BUD-4, and the localization of both proteins lead to the recruitment of RHO-4. Moreover, the localization of BUD-3 and BUD-4 as cortical ring requires RHO-4, providing a potential positive feedback loop for the stable accumulation of the BUD-3-BUD-4-RHO-4 complex at presumptive septation sites prior to septum constriction (Justa-Schuch *et al.* 2010; see Fig 3 for a simplified model). The expression of an activated (GTP-hydrolysis defective) allele of RHO-4 generates increased numbers of actin rings, indicating that RHO-4-GTP can initiate CAR formation and constriction (Rasmussen & Glass 2005). Intriguingly, RHO-4 activity is also regulated through a second RHO-4-specific GEF, RGF-3, which is essential for septum formation and functions in a non-redundant manner with BUD-3 downstream of BUD-3 (Justa-Schuch *et al.* 2010). The cortical localization of RGF-3 requires the presence of BUD-4 and the BUD-3-RHO-4 module, but is not dependent on BNI-1, suggesting that the RGF-3-RHO-4 module functions downstream of the BUD-4-BUD-3-RHO-4 module, but upstream of BNI-1, potentially in mediating formin recruitment to the site of CAR assembly. RHO-4 is negatively regulated by the sole GDP-disassociation-inhibitor RDI-1 (Rasmussen & Glass 2007). Δ*rdi-1* mutants have shorter hyphal compartment lengths, but the appearance of septa is normal. Excessive membrane localization of RHO-4 in Δ*rdi-1* indicates that RDI-1 acts to remove RHO-4-GDP from the plasma membrane. Intriguingly, RHO-4 localization in conidia is mostly cytoplasmic in a *wild-type* background, whereas RHO-4 is concentrated at the plasma membrane in

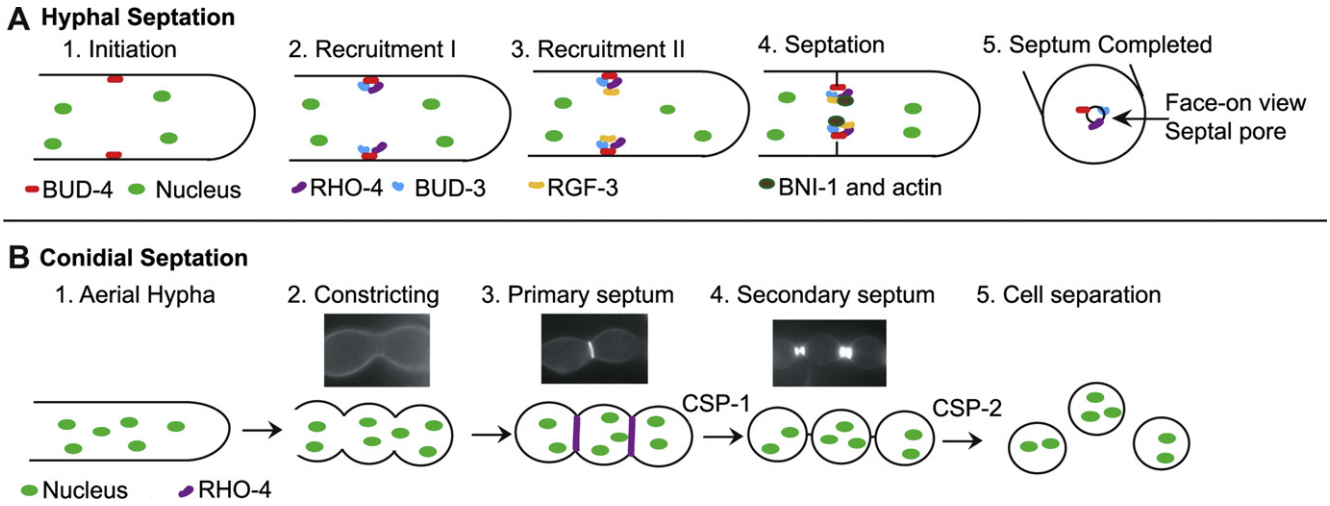

Fig 3 – Model for septum formation during hyphal growth (A) and conidia formation (B). During hyphal septum formation an ordered recruitment of proteins (BUD-4, RHO-4, BUD-3 and RGF-3) to the site of septum formation is followed by the invagination of the plasma membrane, led by an acto-myosin contractile ring.

Δ*rdi-1*. Thus RDI-1 may control septum initiation by regulating the level of membrane-associated RHO-4.

The actin organization during the septation process was recently studied by two groups, using a set of GFP-fusion proteins attached to different actin-binding proteins/domains (Berepiki *et al.* 2010; Delgado-Alvarez *et al.* 2010). Although all constructs transiently labelled the forming septum, distinct temporal and spatial localization patterns suggest functionally distinct F-actin populations during septum formation. Lifeact-GFP, which labels both actin patches and cables, appears ca. 4 min prior to membrane invagination as broad network and cables that coalesce ca. 2 min later into one distinct cortical ring. After the start of constriction (time point 0), lifeact-GFP labels a single constricting ring that disappears from the septal pore ca. 20 min after initial membrane constriction. In contrast, tropomyosin-GFP, a marker for F-actin cables, appears much later and only a few seconds prior to membrane invagination as a sharp cortical ring that constricts and disappears already 9 min after constriction initiation from the spetal pore. Fimbrin-GFP and ARP2-3-GFP, marker proteins for actin patches that are critical for endocytosis and membrane recycling, both appear at the time of membrane invagination as a double ring of patches flanking the invaginating membrane, suggesting membrane turnover (e.g., during the recycling cell wall polymerizing machinery) in the later stages of septum formation.

The cell wall of septa has a different structure and composition that the hyphal lateral wall (Hunsley & Gooday 1974). Live imaging has shown that all seven chitin synthases reported in *N. crassa* localize at nascent septa (Riquelme *et al.* 2007; Sanchez-Leon *et al.* in press; Fajardo-Somera, unpubl. data), whereas no glucan synthase regulator was found at forming septa (Verdin *et al.* 2009). This agrees with early studies showing that *N. crassa* septa are predominantly comprised of chitin (Hunsley & Gooday 1974).

Two other aseptate mutants have been identified in *N. crassa*, *cwl-1* and *cwl-2* (Garnjobst & Tatum 1967; Raju 1992). These mutants are both located on chromosome 2, but the genes responsible have not yet been identified. CWL-1 likely acts downstream of RHO-4, because RHO-4 still localizes to presumptive future septation sites in *cwl-1* mutants. In contrast, RHO-4 does not form rings in the *cwl-2* mutant, placing CWL-2 upstream of RHO-4 (Rasmussen & Glass 2007).

In contrast to the SIN and RHO-4 modules, which when mutated result in aseptate strains, several conditional mutants were identified that generate increased numbers of septa, identifying their gene products as negative regulators of septum formation (Seiler & Plamann 2003). Most notably are LRG-1, a RHO-1-specific GTPase activating protein (GAP; Vogt & Seiler 2008) and COT-1, POD-6 and two MOB-2 proteins, the central elements of a morphogenesis-related NDR (nuclear Dbf2p-related) kinase network (Yarden *et al.* 1992; Seiler *et al.* 2006; März *et al.* 2009; März & Seiler 2010). LRG-1, COT-1 and POD-6 localize to forming septa, providing additional support of their function during septation, but mechanisms of their function during septation have not yet molecularly characterized. Interestingly, *lrg-1* and *cot-1* or *pod-6* mutants display genetically synthetic interactions as do *cot-1* and mutants defective in SIN pathway components (Seiler & Plamann 2003; März *et al.* 2009). A connection between Rho1p and Cbk1p (the homologous NDR kinase of budding yeast) was also described in *Saccharomyces cerevisiae* (Schneper *et al.* 2004). Moreover, a direct inhibitory function of the SIN on the *S. pombe* NDR kinase Orb6 was recently demonstrated (Ray *et al.* 2010). An intriguing hypothesis would thus place RHO-1 in parallel to RHO-4 signalling in jointly regulating the BNI-1-dependent initiation of CAR formation and the COT-1 complex downstream of and negatively regulated by the SIN.

Morphology and function of the mature septum

The septa of *Neurospora crassa* and other Pezizomycotina species are generally perforated by simple pores of 350–500 nm in diameter, which allow nuclei, organelles and cytoplasm to move between compartments. The structure and composition of the septa in *N. crassa* varies with increasing age. Septa are often plugged in older mycelia and upon hyphal injury (Trinci &

Collinge 1973; Hunsley & Gooday 1974). A major component of this septal plug is, as mentioned above, the WB (Tenney *et al.* 2000; Jedd & Chua 2000). Intriguingly, SOFT, a Pezizomycotina-specific protein that is involved in cell–cell signalling during cell fusion (Fleißner *et al.* 2009b) localizes to the septal plugs in *N. crassa* and other filamentous ascomycetes. This septal plug association of SOFT is independent of the WB and aids in sealing of the septal pore (Fleißner & Glass 2007; Maruyama *et al.* 2010), potentially indicating that the signalling machinery of the cell fusion pathway is also associated with the septum.

Additional evidence for septal pores as signalling hubs is supported by the persistence of the BUD-4-BUD-3-RHO-4 module at septal pores (Justa-Schuch *et al.* 2010; Rasmussen & Glass 2005, 2007). A possible function of RHO-4 at the septal pore may be sensing compartment ends and/or length by modulating cytosolic microtubule organizing centers (MTOCs) that are associated with mature septa. Although the existence of such cytosolic MTOCs is not yet confirmed in *N. crassa*, they were recently described in *Aspergillus nidulans* (Veith *et al.* 2005; Xiong & Oakley 2009; Zekert *et al.* 2010). In line with this hypothesis, *rho-4* mutants displayed altered microtubule dynamics, and almost all MTs originate from nuclear spindle pole bodies (Rasmussen *et al.* 2008).

Septum formation during development

The process of asexual spore (conidia) formation in *Neurospora crassa* and other filamentous fungi is analogous to cell separation in unicellular yeasts and requires the digestion of the primary cell wall material between two completely formed secondary septa to release mature spores (Springer & Yanofsky 1989). Mutants of all currently characterized proteins required for septum formation are aconidiate (e.g., the SIN mutants *Δdbf-2* and *Δmob-1*, and RHO-4 module mutants *Δrho-4*, *Δbud-3*, *Δbud-4* and *Δrgf-3*; März *et al.* 2009; Rasmusen & Glass 2005; Justa-Schuch *et al.* 2010; Dvash *et al.* 2010). Thus the functionality of the SIN pathway and of RHO-4 signalling is absolutely required for conidiation. Interestingly, RHO-4 function is not required for conidiation in an adenylate cyclase (*cr-1*) mutant background, and a *rho-4;cr-1* double mutant forms conidiophores. However, the *cr-1; rho-4* conidia seem partially blocked in primary septum formation (Rasmussen & Glass 2007). RHO-4 localizes cytoplasmically just prior to conidial separation, potentially through its cytosolic sequestration *via* interaction with its negative regulator, RDI-1 (Rasmusen & Glass 2007). This localization pattern in addition to analysis of the *cr-1; rho-4* double mutant suggests that RHO-4 may function during primary septum formation, but may not be required for the final step of conidial separation.

Sexual development is also affected in these aseptate strains. They are female sterile and do not form protoperithecia. Moreover, homozygous crosses of Δ*rho-4*, Δ*dbf-2* and Δ*mob-1* mutants, in which the female partner has been sheltered by a helper strain, are barren and produce very few ascospores. Interestingly, no septa are formed in ascogenous hyphae in these mutants, indicating multiple developmental defects of theses strains (Rasmussen & Glass 2005; März *et al.* 2009). Defects in perithecial development accompanied by an increase in septation frequencies have been observed in the *N. crassa snt-2* mutant, defective in a BAH/PHD-containing transcription factor (Denisov *et al.* 2011). Furthermore, inactivation of *snt-2* is accompanied by a significant increase in the autophagy-related *idi-4* gene, suggesting a possible link with the target of rapamycin (TOR) kinase pathway, in *N. crassa* as well as in *Fusarium oxysporum*.

Even though our understanding of septum formation is expanding, the possible connection between tip extension, nuclear behaviour and cortical landmark proteins during septum placement remains unclear. The nature of the link between the SIN and COT-1 pathways has yet to be determined as are specific questions concerning the function(s) of the anillin scaffold and the RHO-1 and RHO-4 GTPase modules during CAR positioning and assembly and what are the functions of the landmark proteins at mature septal pores. Lastly, is the formation/maintenance of all septa commonly regulated and, specifically, are there differences in vegetative *versus* sexual developmental stage-associated septa?

The molecular basis of branching

The exponential growth of a fungal colony by polar tip growth and the generation of new tips through formation of branches allow for fast coverage and exploitation of potential substrates. In *Neurospora crassa* two distinct types of branches are commonly observed (Riquelme & Bartnicki-Garcia 2004). While apical branching involves a significant disturbance in the growth rate and morphology of the parental hyphal tip, including a temporary disappearance of the Spitzenkörper, lateral branching occurs without any detectable alterations in the growth or Spitzenkörper behaviour of the parental hypha. It is generally believed that formation of lateral branches involves the regulated action of cell wall remodelling enzymes, whereas apical branching occurs as a result of an alteration of the polarization machinery at the apex (Riquelme & Bartnicki-Garcia 2004). One clear indication as to the significance of branching in the growth and development of *N. crassa* (and other filamentous fungi) is the fact that, so far, no mutants that do not branch have been described (Perkins *et al.* 2001; Dunlap *et al.* 2007). However, multiple mutants in which a diverse array of impaired genes/gene products increase the frequency of branching or alter branching patterns have been identified (Gavric & Griffiths 2003; Propheta *et al.* 2001; Resheat-Eini *et al.* 2008; Seiler & Plamann 2003; Borkovich *et al.* 2004). Moreover, branch formation can be influenced by multiple environmental factors including temperature, light, physical perturbation/damage, changes in nutrient source as well as by adjacent hyphae (Lauter *et al.* 1998; Watters *et al.* 2000; Watters & Griffiths 2001; Glass *et al.* 2004; Harris 2008).

The emergence of a new branch requires the establishment of a new axis of cell polarity and the subsequent cytoskeleton-dependent transport (Riquelme & Bartnicki-García 2004; Mouriño-Pérez *et al.* 2006) of material to this site for sustained tip growth (e.g., enzymes, membranes and cell wall precursors). Theoretically, the selection of a new branch site could be a purely stochastic process dependent on spontaneous polarization as has been observed in other model systems (Altschuler *et al.* 2008; Slaughter *et al.* 2009). This is supported by analysis of various hyperbranching mutants, demonstrating that the position of branch sites is not preselected and that branching can occur at any position within a hypha. This hypothesis dates back to pivotal physiological studies

originating in filamentous fungi, including *N. crassa*, proposing that a new branch is induced at random position, when the cellular biosynthetic capacity exceeds a certain threshold (Robertson 1959; Trinci 1969; Katz *et al.* 1972). The fact that distances between branches can vary immensely (from a few μm to >1000 μm with the most common branch interval length of around 100 μm) further supports the possibility that branches can be formed at almost any hyphal position.

A variety of mutants also indicate that the position of branch sites is not preselected and that branching can occur at any position within a hypha. Several mutants were isolated that are blocked at distinct steps during branch emergence (Seiler & Plamann 2003), indicating that branch formation is a genetically separable process, consisting of at least four discrete steps: (i) the selection of a new branch site, (ii) the broadening of this spot into a zone of growth, (iii) the production of a short stalk-like branch, and (iv) a maturation step involving microtubules. The initiation of growth requires signal transduction by Rho-type GTPase and NDR kinase pathways and enzyme-dependent cell wall remodelling (and, most likely, additional components). Interestingly, the further characterization of the NDR kinase COT-1 revealed that, although this kinase controls both apical tip extension and branch formation, these two functions can be separated by modulating kinase activity (Ziv *et al.* 2009). This independence of tip extension rate and branching is also supported by inhibitor approaches (Pereira & Said 2009).

GFP technology and live imaging revealed that the polarisome component SPA-2 may be involved in marking the site of lateral branch emergence (Araujo-Palomares *et al.* 2009). Fractions of SPA-2-GFP detached from the parental polarisome, thereby displacing the original polarisome from its typical central position, which may serve as a mark for the site of new branch emergence. However, *Δspa-2* forms more, instead of less, branches, an example of the difficulty in interpreting the functional significance of the observed localization of a specific protein. Moreover, other Spitzenkörper and exocyst components labelled with fluorescent proteins localize at lateral branch sites after a new branch has emerged (Riquelme *et al.* 2007; Verdin *et al.* 2009), suggesting their assembly at the branch point as a consequence of the polarization event.

Taken together, despite the continuous observations of branching processes and patterns in *N. crassa*, our fundamental understanding of the mechanisms involved are still limited. We have yet to determine whether specific landmark proteins determine branch site selection and whether expression/localization/activation of such proteins is linked with nuclear and septum position and cell cycle. Once a branch position has been determined, how is the function of biosynthetic/degradative machinery involved in branch formation balanced and what is the checkpoint defining the transition from an emerging branch to a hyphal cell?

Importance of nucleus architecture, movement and positioning for cell function: from germling to 'colony'

Studies on stage-specific transcription (Sachs & Yanofsky 1991; Kasuga & Glass 2008) indicated that transcript profiles can change drastically, and thus, likely, changes in nucleus architecture (i.e., relative position of active and silent regions of chromatin with respect to the nuclear membrane) are expected to occur during these first hours of mycelium development. In the past studies focused on early development, i.e., after germ tubes become established (~4–6 h of incubation), or on exponentially growing mature leader hyphae and their behaviour. What has emerged from recent studies with fluorescently labelled proteins is that *Neurospora crassa* undergoes a developmental switch along this timeline, largely marked by the emergence of the Spitzenkörper (see above).

In contrast to many other filamentous fungi (Xiang & Fischer 2004; Gladfelter & Berman 2009), daughter nuclei that result from mitoses in rapidly expanding mycelia of *N. crassa* do not usually remain in the same compartment. They travel long distances through septal pores (Freitag *et al.* 2004). Dynein is central to nucleus positioning and migration, at least in the early phase of colony establishment, was shown by studies of *Aspergillus nidulans nud* (nuclear distribution) and *N. crassa ropy* mutants. Several *nud* and *ro* genes encode dynein or dynein-interacting proteins, and in *N. crassa* (Plamann *et al.* 1994; Minke *et al.* 1999a, b), as in *A. nidulans* (Xiang *et al.* 1994) and *Ashbya gossypii* (Alberti-Segui *et al.* 2001), nuclei are unevenly spaced and tend to form clumps when dynein is absent or mutated.

Thus, in the early *N. crassa* germling, nucleus positioning and transport appear to be active processes, dependent on dynein (Plamann *et al.* 1994; Minke *et al.* 1999a, b). In *A. nidulans*, dynein appears also required for even spacing of nuclei throughout the mature hypha and indeed the whole mycelium (Xiang & Fischer 2004). It remains unclear if this is based on dynein's function as a motor or on its effect on MTs (Riquelme *et al.* 2000; Xiang & Fischer 2004). At any rate, this even spacing exists in *N. crassa* only in early germlings and is entirely lost in mature hyphae. Apically extending hyphae carry nuclei forward, in older colonies increasingly aided by cytoplasmic flow of nuclei that appear to be 'trapped' in the cytoskeletal network, perhaps mostly MTs (Mouriño-Pérez *et al.* 2006; Ramos-García *et al.* 2009). At this stage, active anterograde and retrograde transport of nuclei appears to be dependent on actin MFs and MTs, as suggested by experiments with cytochalasin A and benomyl, respectively (Ramos-García *et al.* 2009). From these and related studies (Riquelme *et al.* 2000, 2002) it is clear that dynein does not simply affect nucleus positioning and migration but is also involved in overall architecture of the hypha. Separating dynein's effect on nucleus positioning and the general architecture of the hypha remains a challenge for the future.

If the developmental program from *N. crassa* conidia to fast-growing mature leading hyphae is to be fully uncovered, there is an urgent need to expand our understanding of the genetics and biochemistry of early mitospore or meiospore germination. As far as nucleus positioning in these early events is concerned, why does dynein seem important early on in development but not later in the quickly expanding mycelium? This question clearly deserves renewed attention, especially in light of interesting new results from studies of dynein and its associated proteins in neurons (Tsai *et al.* 2010; McKenney *et al.* 2010; Mao *et al.* 2010).

Similarly, how and when mitosis occurs in *N. crassa* is woefully understudied, in stark contrast to *A. nidulans*. Modern imaging and modelling tools, however, now allow the capture of

mitoses in *N. crassa* hyphae (Freitag *et al.* 2004; Roca *et al.* 2010; Angarita-Jaimes *et al.* 2009) and may uncover rules for mitosis with regard to positioning in the growing hyphae. Incorporation of the use of temperature-sensitive (ts) nuclear cycle mutants (see above, section on Nuclear behaviour and cortical landmark proteins may specify septum placement) would certainly expand the capabilities of studying the processes involved. The single ts nucleus division cycle mutant isolated in *N. crassa*, *ndc-1* (Serna & Stadler 1978), is arrested at the stage of SPB duplication when shifted from 25 °C to 32 °C. In contrast to earlier reports (Serna & Stadler 1978), this mutation is not ts-lethal, as shifting the strain back from 32 °C or even 37 °C to 25 °C rescues the defect (P. Phatale, R. Ramirez-Cota & M. Freitag, unpubl. data). Mapping *ndc-1* by bulk segregant analyses followed by high-throughput sequencing revealed a single point mutation in *spe-1*, the gene encoding ornithine decarboxylase, ODC, the rate-limiting enzyme in polyamine biosynthesis (K.R Pomraning, K.M. Smith and M. Freitag, submitted). There is precedence for this observation, as ODC has been previously found to be involved in yeast and human cell cycle control (Mäkitie *et al.* 2009; Schwartz *et al.* 1995).

A second promising approach to understand differences in the regulation of mitosis between *A. nidulans* and *N. crassa* is study of the nuclear pore complex (NPC), as recently carried out with great success in *A. nidulans* (Liu *et al.* 2009). A similarly sweeping study is currently lacking for *N. crassa*. Nevertheless, the localization studies of a single component of the NPC, SON-1, revealed that *N. crassa* carries out a truly closed mitosis, perhaps similar to that found in yeast (Roca *et al.* 2010). This was predicted from *N. crassa*'s asynchronous mitoses – an open mitosis would allow signalling molecules to access all nuclei similarly and initiate mitosis in a synchronous manner (De Souza & Osmani 2007). Neither the nuclear membrane nor NPC, imaged by presence of SON-1-GFP, broke down when germlings were followed through mitotic cycles (Roca *et al.* 2010). In contrast, the *A. nidulans* NPC partially disassembles, which results in release of the SON-1 homologue, SonB, from the NPC (De Souza *et al.* 2004). Thus, as in many eukaryotes where both the nuclear membrane and the NPC break down, Aspergillus is expected to have fewer – if any - regulatory steps that involve transport of proteins or RNA into the nucleus upon division (De Souza & Osmani 2007, 2009). *Neurospora crassa*, on the other hand, is expected to make use of specific macromolecules to allow import of proteins or RNA through an active and selective NPC, which signals onset of mitosis in each individual nucleus. It is presently a mystery how this can be achieved in a syncytial hypha. In addition to *N. crassa*, *Aspergillus gossypii* (Gladfelter *et al.* 2006) promises to be a tractable system that should shed light on this aspect of hyphal biology.

Does nucleus architecture reflect cell state?

That *Neurospora crassa* chromatin structure seems responsive to the direction of nucleus migration was first shown in studies with GFP-tagged versions of the linker histone H1 (Freitag *et al.* 2004a) and Heterochromatin-binding Protein 1, HP1 (Freitag & Selker 2005). In these images, the centromere-associated heterochromatin appeared to travel at the leading edge of the nucleus, which in turn appeared to be attached to the microtubule network. Polarization of histone localization was also observed in migrating mammalian cells in tissue culture suggesting that some coordination between chromatin, nucleus and cell migration may be conserved (Gerlitz *et al.* 2007). How precisely chromatin, the kinetochore, SPB and MTs are organized at the nuclear membrane in interphase is unresolved for *N. crassa*. In fission yeast, the model organism most closely related to *N. crassa*, electron microscopy (Kniola *et al.* 2001) combined with incisive genetic analyses (Alfredsson-Timmins *et al.* 2007; King *et al.* 2008), suggests that heterochromatin surrounding the centromeric DNA is directly attached to a number of protein complexes that help to anchor this domain to the nuclear membrane. Studies of various components of the heterochromatin-centromere-kinetochore-MTOC 'sandwich' suggest that the arrangement may indeed be similar in *N. crassa* and that it is dependent on proper heterochromatin assembly (P. Phatale, R. Ramirez-Cota, L. Sanchez-Hernandez, M. Riquelme, R. Mourino-Perez & M. Freitag, unpubl. data). This brings up the question as to whether MTs and the hypha direct the nuclei or whether instead there may be a heterochromatin- or perhaps more generally chromatin-dependent checkpoint at work in *N. crassa* that controls hyphal growth. Mutants defective in DIM-5, the histone H3 lysine 9 trimethylase (H3K9me3), showed variable growth defects (Tamaru & Selker 2001), including 'Start-Stop' behaviour. In such strains, HP1, the protein that recognizes H3K9me3, is almost completely mislocalized from heterochromatin, and these strains show extreme growth defects (Freitag *et al.* 2004b), which suggests presence of either the above-mentioned checkpoint or chromosomes segregation defects that result in few viable nuclei. How these very sick HP1 mutants gradually aquire almost normal growth (M. Freitag & E.U. Selker, unpubl. data) is a mystery that likely connects epigenetic control of gene regulation to the control of hyphal growth.

What then are some of the proteins that can effect coordination of heterochromatin and MTs? In fission yeast, just like in flies and mammals (Razafsky & Hodzic 2009; Stewart-Hutchinson *et al.* 2008; Crisp *et al.* 2006), LINC complex proteins have been identified. Fission yeast proteins embedded in the nuclear envelope couple cytoplasmic MTs mechanically to heterochromatin (King *et al.* 2008). One is an integral outer nuclear membrane protein of the KASH family, Kms2, and two are integral inner nuclear membrane proteins, the SUN-domain protein Sad-1 and Ima1, which specifically binds to heterochromatic regions and promotes tethering of centromeric DNA to the SUN-KASH complex. *Neurospora crassa* has putative homologues of two of these proteins, all of which are not very well conserved across eukaryotes and thus need likely to be discovered by forward genetic screens or biochemical methods (P. Phatale & M. Freitag, unpubl. data). At least in fission yeast, Ima1 and the centromeric Ndc80 complex are required for efficient coupling of centromeric heterochromatin to Sad-1 (King *et al.* 2008; not to be confused with *N. crassa* SAD-1, a protein involved in meiotic silencing). Defects result in striking inability of the nucleus to tolerate microtubule-dependent forces. Whether this is similar in any filamentous fungus remains to be seen, but preliminary results suggest that this is the case (P. Phatale & M. Freitag, unpubl. data).

Hyphal tropisms

Colony establishment and development within specific habitats require individual hyphae to re-orient tip growth in response to environmental cues. Examples of such tropic reactions include mutual avoidance of hyphal tips at the periphery of the growing colony, orientation of trichogynes towards a source of mating pheromone or mutual attraction between fusion hyphae or germinated conidia during anastomosis formation. Already early mycologists were intrigued by their observations of these tropic responses, prompting them already to propose the existence of chemoattractants, which support vegetative and sexual development (Ward 1888; Backus 1939).

On the molecular level directed growth relies on the interplay of signal recognition pathways with the general machineries controlling polarity establishment and hyphal tip extension. The direction of tip growth is controlled by the position of the Spitzenkörper (Girbardt 1957: Riquelme *et al.* 1998) a conclusion vividly supported by laser manipulation of the Spitzenkörper (Bracker *et al.* 1997). Thus during tropic responses external signals ultimately have to be translated into re-positioning of this vesicle supply center.

Sexual trichogyne–conidium interactions are controlled by mating type specific pheromones and their respective receptors. In *Neurospora crassa* the *mfa-1* gene encodes the *mat*a pheromone, a 24-residue hydrophobic peptide (Bobrowicz *et al.* 2002; Kim *et al.* 2002). The *mat*A pheromone is encoded by the *ccg-4* gene as a pre-polypeptide, consisting of five repeats of the mature peptide (Bobrowicz *et al.* 2002). These pheromones are essential and sufficient to direct trichogyne growth. Their absence has no obvious impact on vegetative development (Kim & Borkovich 2006). The respective pheromone receptors were identified as PRE-1 and PRE-2, two transmembrane G-protein coupled receptors highly expressed in either *mat*A or *mat*a strains, respectively (Pöggeler & Kück 2001). *Δpre-1* trichogynes are unable to recognize mata cells, thus rendering the mutant female sterile (Kim & Borkovich 2004). While these signalling molecules and the receptors are similar to the pheromone and pheromone receptors mediating mating in *Saccharomyces cerevisiae*, it still remains an open question if homologues of the yeast pheromone response pathway transduce the signal in *N. crassa*. Once trichogyne and conidium have established physical contact, fusion between these two different cell types takes place. Membrane merging seems to be mediated by a general fusion machinery, also involved in other cell fusion events, such as vegetative germling fusion (Fleißner *et al.* 2009a).

In the inner older parts of mature colonies specialized hyphae attract each other ('home') and fuse (Hickey *et al.* 2002). These anastomoses increase interconnectedness within the mycelium and probably support coordinated colony

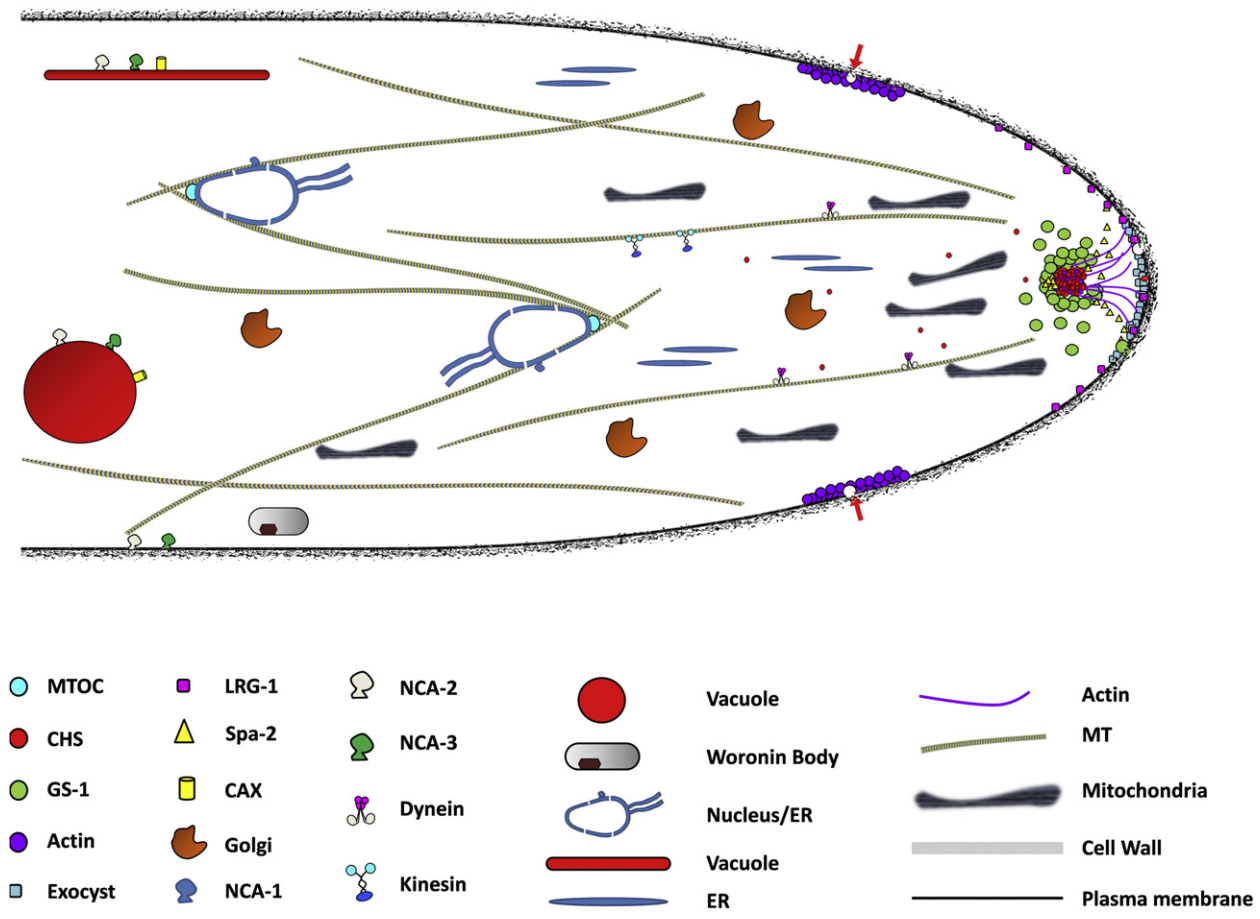

Fig 4 – Overall scheme of a *N. crassa* hyphal tip showing distribution of organelles, cytoskeleton and polarity factors, based on the localization of proteins tagged with fluorescent proteins.

behaviour. Although not discussed in this review, regulation of vegetative incompatibility reactions following hyphal fusion has been extensively studied in *N. crassa* (see reviews by Saupe *et al.* 2000; Aanen *et al.* 2010).

Similar to hyphal fusion conidia and conidial germ tubes of *N. crassa* form specialized fusion structures, so called conidial anastomosis tubes (CATs), which exhibit positive tropic reactions resulting in cell fusion (Roca *et al.* 2005). Within the last few years numerous molecular factors controlling and mediating these vegetative fusion events have been identified (Simonin *et al.* 2010; Aldabbous *et al.* 2010; Fleißner *et al.* 2009a; März *et al.* 2009; Read *et al.* 2010). Recently, an unusual form of cell-to-cell signalling related to germling fusion in *N. crassa* was described (Pandey *et al.* 2004; Fleißner *et al.* 2005). The MAP kinase MAK-2 and the SO protein are both essential for mutual attraction of fusion hyphae. During chemotropic growth both proteins are recruited to the plasma membrane of the hyphal tips in an oscillating alternating manner. While MAK-2 localizes to the tip of the first fusion partner, SO is present at the plasma membrane of the second fusion tip. After a few minutes the roles were reversed and SO is recruited to the apex of the first germling, while MAK-2 accumulates in the tip region of the second fusion cell (Fleißner *et al.* 2009b). These observations suggest that the two fusion partners alternate between two physiological stages in a highly coordinated manner. An attractive hypothesis is that the germlings coordinately switch between signal sending and receiving, thus avoiding self-stimulation: a true cell-to-cell dialogue. The nature of the involved signalling molecules still remains elusive. Mating pheromones and receptors are dispensable for vegetative hyphal fusion, indicating that distinct signalling systems support vegetative and sexual development (Kim & Borkovich 2004, 2006).

Although hyphal tropic responses were already described in the early days of mycology, we are still just at the beginning of understanding their underlying molecular and cellular mechanisms. Many open questions remain, including: How is the detection of environmental cues translated into re-positioning of the tip growth machinery? What are the common and the distinct factors controlling different tropic responses? What role do ion gradients (Ca^{2+}, H^+, Na^+, etc.) play in the signalling process towards the polarity machinery? Which environmental signals control growth directionality during colony establishment and development? What is the nature of the signalling components involved in cell–cell communication during anastomosis formation? What are the molecular mechanisms behind hyphal avoidance reactions? How do specific signalling pathways interact with the general polarity machinery to control tropic responses, such as in trichogyne–conidium interaction or vegetative hyphal fusion and how do these pathways link the sensing modules with the cell wall remodelling machinery?

Conclusions, prospects and open questions

In many ways *Neurospora crassa* has been at the forefront of analyses of the hyphal cell. The continuous progression of fungal research, using *N. crassa* as a model, has not only yielded exciting results (Fig 4) but has also set the stage for future advantageous probing and elucidation of the nature of fungal biology with this organism. The availability of the complete *N. crassa* genome, an almost saturated collection of single-gene deletion mutants, along with the capabilities of transcriptome analysis has opened new possibilities for functional analysis of gene function. Such analyses can provide novel information concerning the requirements for hyphal development, along with the quantitative temporal and spatial kinetics of gene expression through development, and include either the analysis of specific genes of interest (e.g., the *hex-1* gene involved in WB formation; Tey *et al.* 2005) or genome-wide analysis (Kasuga & Glass 2008) of gene expression of developing hyphal structures (or the entire colony). These analyses, combined with high-throughput methods that directly link activity of chromatin-binding transcription factors to expression levels (Smith *et al.* 2010) will pave the way of determining the hierarchical programs governing hyphal development. As much of biochemical cell function is determined by proteins, there is a growing need for progress in fungal proteomics in order to provide even a more comprehensive understanding of hyphal cell development (Kim *et al.* 2007). Additional fields, which have accompanied fungal research, have not yet been completely integrated into current fungal development research to yield the expected impact, e.g., physiology and mathematical modelling. For example, even though historically *N. crassa* was one of the organisms used to discover the central role of the plasma membrane H^+-ATPase in generating the negative-inside electrical potential of plant, algal and fungal cells (Slayman 1965), a prerequisite for cell viability, the mechanistic link between ionic homeostasis with hyphal development has not been fully elucidated. Calcium is one example of an internal developmental cue (for details of Calcium signalling machinery see Zelter *et al.* 2004). Others may exist, but the map is currently almost completely blank. Could inter-organellar ion transport regulate the internal architecture of the hyphal cell and development of the mycelial network? So far, such a role has only been directly identified for tip-localized mitochondria that appear to function in Ca^{2+} sequestration behind the growing tip (Levina & Lew 2006).

There is another state property of the hypha which impacts directly on growth and development: pressure. Here, ion transport plays a crucial role, but in concert with molecular genetics *via* signalling kinase cascades. The osmotic MAP kinase cascade activates not only upregulation of glycerol biosynthesis genes (Noguchi *et al.* 2007) but also activation of the plasma membrane H^+ pump to drive uptake of ions, thereby regulating turgor in response to hyperosmotic shock (Lew *et al.* 2006). Separately, intra-hyphal pressure gradients cause the mass movement of cytoplasm within the mycelial network (Lew 2005; Ramos-García *et al.* 2009). Here, hyphal development relies upon 'action at a distance': long distant transport that is caused by relatively small differences in osmotic pressure. In fact, the concept of 'long-distance' polarity is supported by experiments showing that ionic currents surrounding the hypha exhibit polarized regions of inflow and outflow (Gow 1984; McGillivray & Gow 1986). Internal potential gradients, tip-positive, have also been documented (Potapova *et al.* 1988). Whether or not these polar currents and potential gradients are causes (or effects) of polarized development remains unclear, and won't be elucidated until technical tools allowing their direct manipulation become available.

Mathematical modelling of hyphal growth has accompanied fungal research from its early phases giving us novel insights. By computer modelling and mathematical analysis, the Spitzenkörper was predicted to function as a vesicle supply center (Barnicki-Garcia *et al.* 1989). Equations describing the polarized migration of surface-building vesicles generated realistic hyphal shapes in 2D and 3D (Gierz & Bartnicki-Garcia 2001). But the ultimate validity of the VSC hypothesis depends on demonstrating that the flow of wall-building vesicles passes through a Spitzenkörper control gate. Such traffic of vesicles in/out of the Spitzenkörper is yet to be demonstrated and measured. A mathematical analysis of cytoplasmic events accompanying branching may also lead to a deep understanding of its causes.

Lastly, given the complexity of the growing hypha, can such a complex developmental process be dissected solely on the basis of a reductionist approach? Is there a necessity to revisit or initiate the incorporation of more holistic approaches using mathematical modelling combined with full genome transcription/proteomic approaches in order to obtain 'systems biology'-based answers to the questions posed (Lazebnik 2002; Strange 2005)? Furthermore, non-destructive and non-disruptive measuring and sampling techniques need to be developed or adapted to complement the accumulating genetic and protein-based data? Lastly, given the possibility to accumulate information, are we now in a new era of data collection, which still awaits more involvement of additional scientific disiplines in order to redirect some of our hypothesis-driven research to novel forms of experimentation?

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