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Review

The mechanistic basis of self-fusion between conidial anastomosis tubes during fungal colony initiation

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ABSTRACT

The main model for studying the mechanistic basis of hyphal self-fusion is the conidial anastomosis tube (CAT) system of *Neurospora crassa*. CATs are specialized cell protrusions/short hyphae produced during colony initiation. They grow chemotropically towards each other and fuse to form interconnected networks of conidial germlings. CAT fusion in *N. crassa* is an excellent model for hyphal fusion because it is easy to analyse by live-cell imaging and is well suited for mutant analyses and experimental perturbation using pharmacological agents. ~ 40 mutants compromised at different stages of CAT fusion have been characterized. The CAT inducer and chemoattractant are, as yet, unidentified but have been proposed to be the same self-signalling peptide. CAT fusion requires F-actin but not microtubules, and the polarisome protein complex plays an important role in cell polarity regulation during different stages of the process. Self-signalling, in which genetically identical CATs recognize each other as different, involves what has been coined the 'ping–pong mechanism'. This entails two CATs repeatedly switching their physiological states by the oscillatory recruitment of the proteins MAK-2 and SO to CAT tips as they grow chemotropically towards each other. Once CATs make contact they adhere and the intervening cell wall is remodelled and degraded. This is followed by the merging of the two CAT plasma membranes and the formation of a fusion pore that results in cytoplasmic continuity being achieved between the fused CATs. Mutant analyses have implicated a range of other signalling pathways and processes involved in different stages of CAT fusion. These include: the Rho GTPases CDC-42 and RAC-1; the STRIPAK complex; the cell wall integrity MAP kinase pathway; redox signalling; endocytosis; and five transcription factors.

1. Introduction

Most filamentous fungi form complex, supracellular hyphal networks that are central to colony organization and functioning. Hyphal network formation involves the fusion of genetically identical cells or hyphae and is thus termed self-fusion. It typically occurs during colony initiation by the fusion of spore germlings and then later in the mature colony by anastomosing fusion hyphae (Read *et al.*, 2009, 2010) and/or hyphal aggregates (mycelial cords or rhizomorphs, Fricker *et al.*, 2007; Heaton *et al.*, 2012). Cell fusion during colony initiation in probably the majority of filamentous ascomycetes is brought about by short specialized hyphae or cell protrusions called conidial anastomosis tubes (CATs) (Fig. 1) (Roca *et al.*, 2003, 2005a, b; Read *et al.*, 2009, 2010). Self-fusion has also been reported to occur between other types of spore germlings

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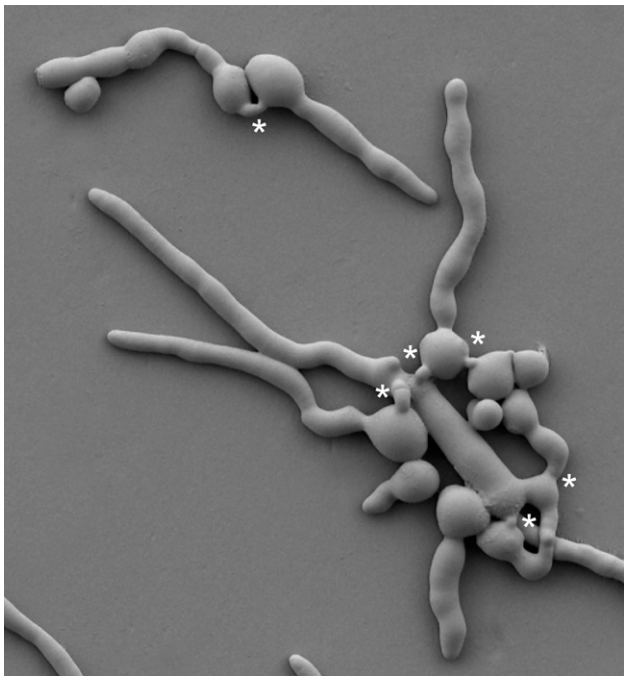

Fig. 1 – Network of conidial germlings fused by CATs (asterisks). Note that germ tubes are wider and longer than the CATs (asterisks) which are short protrusions that have grown towards each other and fused. Image produced by low-temperature scanning electron microscopy (from Roca et al., 2005a with permission).

(e.g. between uredospore or ascospore germlings, Read and Roca, 2006).

2. The CAT system as a model for studying hyphal fusion

The CAT system in *Neurospora crassa* is providing an excellent model with which to analyse the mechanistic basis of self-fusion between fungal spore germlings or hyphae (Roca *et al.*, 2005a; Read *et al.*, 2009, 2010). Although originally described in the plant pathogen *Colletototrichum lindemuthianum* (Roca *et al.*, 2003), the process of CAT fusion has been primarily analysed in *N. crassa* because it exhibits a number of important advantages, including:

(1) CAT fusion is readily monitored by light or fluorescence microscopy and the whole process can be analysed in <6 h (Roca *et al.*, 2005a).
(2) It is easy to screen for mutants defective in CAT fusion (Table 1). This is very much facilitated by the large collection of *N. crassa* mutants available from the Fungal Genetics Stock Center (FGSC; www.fgsc.net). In particular, the FGSC possesses deletion mutants for the majority of *N. crassa* genes that have been generated as part of the *Neurospora* genome project (Park *et al.*, 2011; http://www.dartmouth.edu/~neurosporagenome/proj_overview.html).
(3) Techniques for live-cell imaging of different stages of CAT fusion using various microscopic techniques, coupled with labelling different cell components and molecules with fluorescent proteins and vital dyes, are now well developed (e.g. Roca *et al.*, 2005a, 2010; Fleißner *et al.*, 2009a; Lichius *et al.*, 2012).
(4) The process of CAT fusion is very amenable to experimentation involving the application of chemical inhibitors and agonists (e.g. Fleißner *et al.*, 2009b; Roca *et al.*, 2010). Recently we have found that this type of experimental approach is expedited by using a microfluidic slide culture system (http://www.cellasic.com/ONIX_yeast.html) in which fusing conidial germlings can be immobilized whilst being exposed to growth medium and/or drugs applied by continuous perfusion at particular time points for defined periods of time (Muñoz, A. and Read, N.D., unpubl.).

The CAT fusion process can be conveniently divided into the following continuum of developmental stages that are summarized in Fig. 2: CAT induction; CAT chemotropism; cell–cell adhesion; cell wall remodelling/degradation; plasma membrane merger; and achievement of cytoplasmic continuity between fused CATs (Read *et al.*, 2009).

3. Mutant screening and analysis

Mutant screening has revealed a large number of mutants that are defective in different aspects of CAT fusion (Table 1). To date, all described mutants perturbed in CAT fusion are also defective in hyphal fusion in the mature colony. However, a number of mutants have been identified that are apparently required for fusion in the mature colony but not for CAT formation (Fu *et al.*, 2011). This is not surprising as there are some morphological differences between the two processes (Read *et al.*, 2010). The majority of fusion deficient mutants that have been analysed are blocked in CAT induction (Table 1). This makes it difficult to determine whether proteins encoded by these genes are involved in subsequent stages of CAT fusion because downstream morphogenetic events are masked in these mutants. Alternative methods need to be employed to analyse whether these proteins play multiple roles in CAT fusion. A first step is usually to label the protein of interest with a fluorescent protein and perform live-cell imaging to determine its localization at different stages of CAT fusion (Roca *et al.*, 2005a, 2010; Fleißner *et al.*, 2009a; Berepiki *et al.*, 2010; Lichius *et al.*, 2012). Another approach has been to apply drugs that inhibit the activity of specific proteins at different developmental stages (Fleißner *et al.*, 2009a). An emerging conclusion from these studies is that a number of proteins do indeed operate at different stages during CAT fusion. What has also been revealing, however, is the discovery of mutants that are *not* defective in CAT fusion. For instance, these analyses have revealed no evidence for the involvement of either sex pheromones/sex pheromone receptors (Read *et al.*, 2010), cAMP/protein kinase A signalling (Lichius *et al.*, 2010) or histidine kinase signalling (Heron, K. and Read, N.D., unpublished) in CAT fusion. It should also be noted that a significant number of mutants undergo CAT fusion more slowly than the wild type (Lichius, 2010) but whether any of the defective genes in these mutants are directly involved in the process is unclear at this stage.

Table 1 – Genes involved in different stages of CAT fusion in *N. crassa* as revealed from mutant analyses.

Locus	Gene	Developmental stage that mutant is defective					References
		Type of protein	CAT induction	CAT chemoattraction	CAT fusion	Stage not determined	
adv-1	NCU07392	Transcription factor	●				Fu *et al.* (2011)
ada-3	NCU02896	Transcription factor	●				Fu *et al.* (2011)
amph-1	NCU01069	Amphiphysin	●				Fu *et al.* (2011)
cdc-24	NCU00957	Guanine exchange factor	●				Lichius (2010)
cdc-42	NCU06454	Rho GTPase	●				Lichius (2010)
gpig-1	NCU09757	GPI anchor protein				●	Bowman *et al.* (2006)
gpip-1	NCU06663	GPI anchor protein				●	Bowman *et al.* (2006)
gpip-2	NCU07999	GPI anchor protein				●	Bowman *et al.* (2006)
gpip-3	NCU06508	GPI anchor protein				●	Bowman *et al.* (2006)
gpit-1	NCU05644	GPI anchor protein				●	Bowman *et al.* (2006)
ham-2	NCU03727	Trans-membrane protein	●				Xiang *et al.* (2002); Roca *et al.* (2005a); Fu *et al.* (2011)
ham-3	NCU08741	Striatin	●				Simonin *et al.* (2010); Fu *et al.* (2011)
ham-4	NCU00528	Coiled coil, trans-membrane protein				●	Simonin *et al.* (2010); Fu *et al.* (2011)
ham-5	NCU01789	WD40 domain protein	●				Aldabbous *et al.* (2010); Fu *et al.* (2011)
ham-6	NCU02767	Small hydrophobic protein	●				Fu *et al.* (2011)
ham-7	NCU00881	GPI anchor protein	●				Fu *et al.* (2011)
ham-8	NCU02811	Trans-membrane protein	●				Fu *et al.* (2011)
ham-9	NCU07389	Plekstrin domain protein	●				Fu *et al.* (2011)
ham-10	NCU02833	C2 domain protein	●				Fu *et al.* (2011)
mak-1	NCU09842	MAP kinase	●	●			Lichius (2010); Fu *et al.* (2011)
mak-2	NCU02393	MAP kinase	●				Pandey *et al.* (2004); Roca *et al.* (2005a); Fu *et al.* (2011)
mek-1	NCU06419	MAP kinase kinase	●	●			Lichius (2010); Fu *et al.* (2011)
mek-2	NCU04612	MAP kinase kinase	●				Fu *et al.* (2011)
mik-1	NCU02234	MAP kinase kinase	●	●			Lichius (2010); Fu *et al.* (2011)
lao-1	NCU05113	Ascorbate oxidase-like protein	●	●			Lichius (2010)
mob-3	NCU07674	Phocein	●				Maerz *et al.* (2009); Fu *et al.* (2011)
nor-1	NCU07850	NADPH oxidase regulator	●	●			Lichius (2010)
nox-1	NCU02110	NADPH oxidase	●	●			Lichius (2010)
nrc-1	NCU06182	MAP kinase kinase kinase	●				Pandey *et al.* (2004); Roca *et al.* (2005a); Fu *et al.* (2011)
pkr-1	NCU00506	Assembly factor for V-ATPase	●				Fu *et al.* (2011)
pp-1	NCU00340	Transcription factor	●				Li *et al.* (2005); Read *et al.* (2010)
pp2A	NCU06563	Catalytic subunit of protein phosphatase 2A	●				Fu *et al.* (2011)
pmr-1	NCU09337	Membrane protein	●	●	●		Fleißner *et al.* (2009a)
rac-1	NCU02160	Rho GTPase	●				Lichius (2010); Fu *et al.* (2011)
rcm-1	NCU06842	Transcription factor	●				Aldabbous *et al.* (2010)
rco-1	NCU06205	Transcription factor	●				Aldabbous *et al.* (2010); Fu *et al.* (2011)
snf5	NCU00421	Transcription factor	●				Fu *et al.* (2011)
so	NCU02794	WW domain protein	●	●			Fleißner *et al.* (2005, 2009b); Fleißner and Glass (2007); Fu *et al.* (2011)

● Indicates where developmental blocks occur.

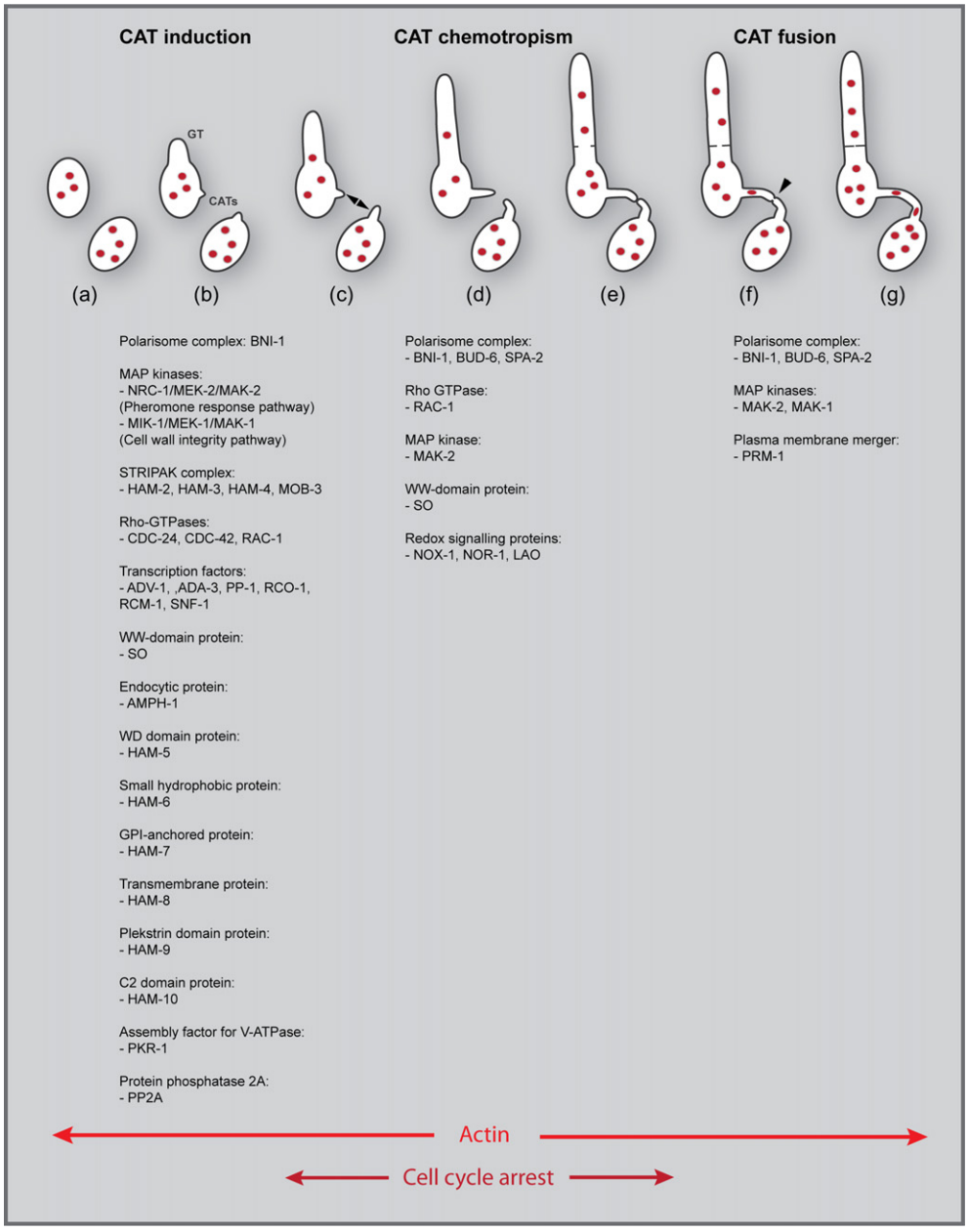

Fig. 2 – Summary of the continuum of developmental stages involved in CAT fusion and the signalling networks and proteins involved. Although not shown here, CATs can either arise from the conidia directly or from germ tubes (Roca *et al.*, 2005a; Read *et al.*, 2009). The three major phases of CAT-mediated cell–cell fusion are CAT induction, CAT chemotropism and CAT fusion. (a) Ungerminated macroconidia contain on average 3–6 nuclei (red circles), and initially grow exclusively by isotropic expansion. (b) Cell polarization leads to the outgrowth of a germ tube (GT) and conidial anastomosis tubes (CATs). (c) Genetically identical cells communicate by releasing an unknown chemoattractant from their tips which is perceived by opposing CAT tips (arrowheads). (d) Orientation of CATs along this chemoattractant gradient results in them growing towards each other to establish cell–cell contact. (e) Upon contact, tip growth arrests and CATs adhere to each other, and this most likely involves adhesive secretion and the remodelling of the cell wall around the contact site in order to prevent leakage during subsequent pore formation. (f) Fusion pore formation (arrowhead) includes localized cell wall degradation and remodelling and the merging of plasma membranes. (g) Upon establishment of cytoplasmic continuity, organelles including nuclei, become mixed between fused germlings. The cell cycle is arrested during CAT chemotropism, and actin, but not microtubules, are required for CAT fusion to occur (Roca *et al.*, 2010).

4. Extracellular self-signalling

CAT induction and CAT chemoattraction are regulated by extracellular signal molecules released by conidia and conidial germlings into their surrounding medium. At present, however, we know neither the identity of these signal molecules nor the receptors that they bind to and activate. Evidence for an extracellular CAT inducer has come from the observation that CAT formation is dependent on conidial density. CATs form when conidia are close together and culture filtrates have been reported to induce CAT fusion in the plant pathogen *Venturia inaequalis*. These observations suggest a possible role for quorum sensing in CAT induction involving individual conidia sensing their density by detecting extracellular chemical signals secreted by the population of conidia (Roca *et al.*, 2005a; Read *et al.*, 2010). Compelling evidence that CATs show mutual attraction towards each other as a result of the release of a CAT chemoattractant has been obtained by using a 'CAT homing assay' involving optical tweezer micromanipulation. When the relative positions of two CATs exhibiting growth towards each other is changed by tweezer micromanipulation then both CAT tips readjust their direction of growth back to towards each other to make contact and fuse at their tips (Fleißner *et al.*, 2005; Roca *et al.*, 2005a; Wright *et al.*, 2007). These results indicate that a diffusible chemoattractant is released from CAT tips and the sensing of this chemoattractant, and thus the activation of its receptors, is located at CAT tips (Roca *et al.*, 2005a).

It is reasonable to propose that individual fungal species only produce a single self-signalling ligand in which the CAT inducer is also the CAT chemoattractant (Read *et al.*, 2009, 2010). This would contrast with the situation of non-self fusion in the budding yeast, *Saccharomyces cerevisiae*, in which two different peptide pheromones that bind to their cognate pheromone receptors on cells of opposite mating type are involved in orchestrating cell fusion. However, each of these sex pheromones is involved in *both* the induction of the cell protrusions (shmoo formation) and in the chemotropism of these protrusions towards each other to effect non-self cell fusion (Read *et al.*, 2009).

cAMP is used as a chemoattractant in cell chemotaxis by the slime mould *Dictyostelium discoideum* (Manahan *et al.*, 2004; Garcia and Parent, 2008; Loomis, 2008). Annotation of the sequenced *N. crassa* genome showed that it encoded three G-protein coupled receptor-like proteins that resembled the cAMP receptors of *D. discoideum* (Galagan *et al.*, 2003; Borkovich *et al.*, 2004). As a result, it was suggested that cAMP, or a related molecule, might act as an extracellular signal in *Neurospora* (Galagan *et al.*, 2003), and thus possibly be a chemoattractant involved in hyphal fusion. However, it was subsequently shown that this was not the case by demonstrating that a *cr-1* mutant lacking cAMP underwent CAT chemotropism and fusion (Roca *et al.*, 2005a). Since conidial germlings of different species do not seem to commonly fuse with each other, although fusion between closely related species has been reported (Köhler, 1930; Roca *et al.*, 2004; Glass and Fleiβner, 2006), a more likely candidate for a CAT chemoattractant is a peptide because this could provide the species specificity necessary to prevent non-self fusion between species (Roca *et al.*, 2005a). Nothing is known about secretion of the chemoattractant although its secretion is believed to be pulsatile (see discussion in Section 6 below).

Other extracellular signals can influence CAT fusion. These can include nutrients (Glass and Fleiβner, 2006; Roca *et al.*, in press), the conidial age (Ishikawa *et al.*, 2010), the growth conditions of the cultures that the conidia are derived from (Roca *et al.*, in press), and the hydrophobicity of the underlying surface (Roca *et al.*, in press). The signalling mechanisms underpinning the influence of these extracellular signals on CAT fusion remain to be elucidated.

5. Cell polarity regulation

Our current view is that the initiation and chemotropic growth of CATs involves cell symmetry breaking and polarized tip growth, that is regulated by the external concentration and gradients of an unidentified self-signalling ligand. In vegetative hyphae, cell-end marker (landmark) proteins, such as Tip Elongation Aberrant (TEA) proteins, mark sites for the establishment and maintenance of tip growth. Evidence indicates that sterol-rich domains in the plasma membrane regulate the positioning of these cell-end markers (Fischer *et al.*, 2008). These features of vegetative hyphae still need to be analysed during CAT induction and growth. The Rho GTPases CDC-42 and RAC-1 become recruited to landmarked plasma membrane regions (Fischer *et al.*, 2008) where their local activity is upregulated through positive feedback mechanisms (Goryachev and Pokhilko, 2008). CDC-42 and RAC-1, and their Guanine Exchange Factor (GEF) CDC-24, have been shown to be essential regulators for the establishment and maintenance of cell polarity in *N. crassa* (Araujo-Palomares *et al.*, 2011). Although the functions of these regulatory proteins have not yet been defined during the process of CAT fusion, Δ*cdc-42*, Δ*rac-1* and Δ*cdc-24* mutants are all defective in CAT induction (Table 1). Effector proteins of Rho GTPases include formins that nucleate F-actin assembly (Goode and Eck, 2007; Chesarone *et al.*, 2010).

A key multiprotein complex involved in regulating the actin cytoskeleton and secretory machinery required for polarized CAT growth is the polarisome. The subcellular organization and dynamics of three polarisome components (BNI-1, BUD-6 and SPA-2) have been analysed at different stages of CAT induction, homing and fusion in *N. crassa* (Lichius *et al.*, 2012). The formin BNI-1, which functions in nucleating the formation of F-actin (Berepiki *et al.*, 2011), accumulates during CAT induction at the site of cell symmetry breaking before the emergence of a CAT. Once a CAT protrusion has been formed the other polarisome components, BUD-6 (an actin interacting protein) and SPA-2 (the central polarisome scaffolding protein) become recruited with BNI-1 to the homing CAT tip. Once CATs make contact they cease to exhibit polarized growth and all three polarisome proteins become concentrated at the site of fusion pore formation. Here they form a ring around the expanding pore which establishes cytoplasmic continuity and then shortly after they disappear from the pore (Lichius *et al.*, 2012; Fig. 3).

CAT fusion requires F-actin but not microtubules. Latrunculin B treatment (which inhibits F-actin polymerization), live-cell imaging of F-actin, and analysis of mutants defective

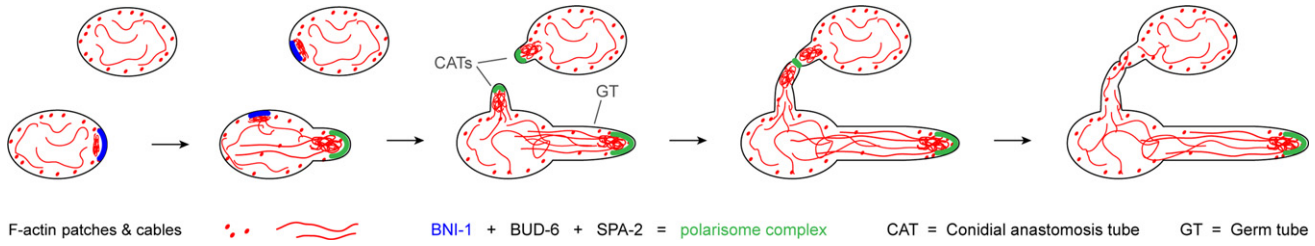

Fig. 3 – Diagram showing localization of F-actin cables and patches, the formin BNI-1 and the full polarisome complex consisting of BNI-1, BUD-6 and SPA-2 during CAT-mediated cell fusion. The formin BNI-1 initiates F-actin assembly at the cell cortex prior to cell symmetry breaking. Dense F-actin cable arrays and the polarisome complex, comprising BNI-1, BUD-6 and SPA-2, accompany polarized protrusion of CATs and germ tubes. Shortly after cytoplasmic continuity is established, polarized CAT tip growth is terminated, the polarisome disassembles, and actin cable arrays dissolve from the fusion site. Polarized tip growth of germ tubes usually commences.

in the Arp2/3 complex (that promotes F-actin assembly, dynamics and possibly crosslinking) demonstrated that F-actin plays important roles in CAT fusion (Berepiki *et al.*, 2010; Roca *et al.*, 2010; Lichius *et al.*, 2011). Treatment with the microtubule depolymerising drug benomyl, on the other hand, did not inhibit CAT induction, homing or fusion. Because the addition of benomyl blocks the formation of elongated germ tubes but does not interfere with CAT-mediated cell fusion, this provides a very useful experimental method for distinguishing CATs from germ tubes, and for discriminating between cell fusion mutants blocked in CAT formation and CAT chemoattraction (Lichius *et al.*, 2010; Roca *et al.*, 2010).

Using the Lifeact peptide reporter fused to GFP or TagRFP, live-cell imaging of F-actin has been possible throughout the whole process of CAT fusion. Actin cables accumulate in the cell cortex at sites of CAT emergence prior to cell symmetry breaking. As the CAT develops, an apical cap of F-actin is formed and remains present in the chemotropically growing CAT tip. Upon CATs making contact with each other, actin cables gradually disappear from the fusion site but some residual cables persist suggesting that they may be involved in a later stage of the fusion process. Once cytoplasmic continuity is fully established, actin cables cease to be present within the fused CATs, whereas cortical actin patches (presumably involved in endocytic recycling) remain (Berepiki *et al.*, 2010; Roca *et al.*, 2010; Lichius *et al.*, 2011; Fig. 3).

To date, no studies have been made of the exocyst protein complex (Jones and Sudbery, 2010) that is involved in regulating secretory vesicle docking and fusion with the apical plasma membrane during CAT induction, chemoattraction and fusion. However, it is clear in *N. crassa* that CATs, like germ tubes, lack the organelle complex called the Spitzenkörper (Read *et al.*, 2010) that is intimately associated with the tip growth and morphogenesis of vegetative hyphae (Girbardt, 1957; Harris *et al.*, 2005; Steinberg, 2007; Jones and Sudbery, 2010).

6. Ping–pong mechanism of CAT chemoattraction

The fundamental basis of how genetically identical CATs can recognize each other as different, and be mutually attracted and fuse, has recently been revealed. It involves the two homing CATs rapidly and repeatedly switching their physiological states as a result of the oscillatory recruitment of different signal proteins to their tips. Two proteins, the MAP kinase MAK-2 and the WW domain containing protein SO, have been shown to exhibit anti-phase oscillatory recruitment such that when MAK-2 is transiently localized to one CAT tip, SO is simultaneously localized in the opposite CAT tip (Fleißner *et al.*, 2009b; Fig. 4). We have coined the term 'ping–pong mechanism' to describe this process because the observed dynamics of MAK-2 and SO indicate that two CATs repeatedly exchange signalling pulses as if batting each other a ball back and forth (Read *et al.*, 2009; Goryachev *et al.*, in press). It has been proposed that one CAT acts as a 'signal sender' and the other as a 'signal receiver' and this alternates back and forth with SO in the signal sending pathway and MAK-2 in the signal receiving pathway (Fig. 4). This pulsatile process is activated when CATs are <15 μm apart and MAK-2 and SO can be repeatedly recruited to a CAT tip 4–6 times during chemotropic growth. Such spatio-temporal coordination of signalling allows the genetically identical and developmentally equivalent CATs to coordinate their behaviour in order to achieve cell fusion, whilst at the same time preventing self-excitation/stimulation (Fleißner *et al.*, 2009b). It has been argued that the period (6–12 min) of MAK-2 oscillation is too short for transcriptional feedback loops to be involved in regulating the ping–pong mechanism (Read *et al.*, 2009). A prediction resulting from the identification of this mechanism is that the CAT chemoattractant released from CAT tips is secreted in a pulsatile fashion (Fleißner *et al.*, 2009b).

The pulsatile communication between CATs has recently been mathematically modelled using a generic activator-inhibitor model of excitable behaviour (Goryachev *et al.*, in press; Fig. 5). The CAT ping–pong mechanism shares features in common with other biological systems that exhibit excitable behaviour (e.g. electrical pulse propagation in nerves, intracellular Ca^{2+} waves, and waves of cardiac muscle contraction). These processes all involve an external stimulus reaching a critical excitation threshold that needs to be exceeded in order to produce the response. This is followed by a refractory period during which the system is unable to respond to another stimulus.

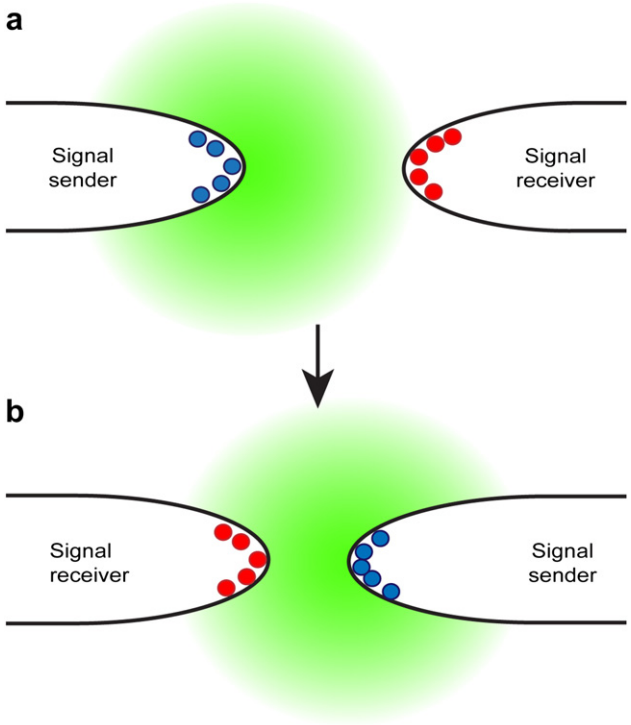

Fig. 4 – Cartoon summarizing the proposed ping–pong exchange of pulses of the unknown chemoattractant. (a) In the first half-period of the signalling, SO protein (blue) is involved in releasing a chemoattractant signal (green cloud) from the left CAT tip, while MAK-2 (red) at the right CAT tip acts in the receiving signal transduction pathway to orientate tip growth towards the chemoattractant source. (b) In the second half period the roles reverse: in the right tip MAK-2 feedback inhibits itself and recruits SO to the apex which releases the next chemoattractant pulse. In the meantime SO has been replaced by MAK-2 in the left tip which is now ready to receive the 'response'. This sequence of pulsatile signalling repeats until both tips come into contact (adapted from Read *et al.*, 2009, with permission).

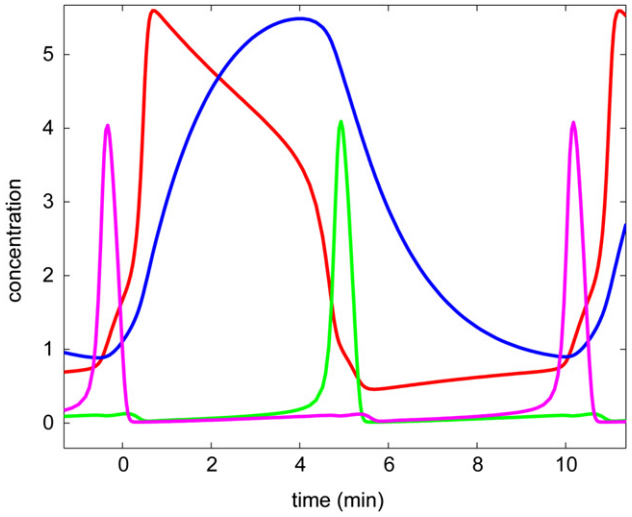

Fig. 5 – Activator-inhibitor model of the ping–pong mechanism of CAT chemoattraction showing one full period of ping–pong dynamics. The time series of the activator (red), inhibitor (blue), and the chemoattractant secretion (green) of one CAT are shown together with the inducing secretory pulses of the opposite CAT (magenta). For details of model parameters see Goryachev *et al.* (in press). It is proposed that the activator might be the active phosphorylated form of the MAP kinase, MAK-2. An inhibitor may not exist. Inhibition of the activator may be achieved by depletion of the substrate that is used up during the activator pulse. It will then need replenishing during the refractory period before the system can undergo another burst of activity (reproduced from Goryachev *et al.*, in press, with permission).

Our model proposes that secretory vesicles containing the chemoattractant molecule are produced at a constant rate by the Golgi, and then transported along actin cables to the CAT tip where they dock at the plasma membrane. After vesicle fusion the chemoattractant can be released into the external medium. In the steady state, when no excitable behaviour takes place, secretion is suggested to occur at a basal rate to enable continuous background signalling. To produce a burst and thus a pulse of secretion, different components of the secretory pathway may be controlled by the activator-inhibitor model. We have postulated that the activator increases the probability of vesicle docking and, at the same time, suppresses their fusion and thus release of chemoattractant. As a result, during the activator pulse, a large number of docked vesicles will accumulate at the CAT tip. Fusion of these vesicles with the CAT tip occurs when the concentration of the activator drops sufficiently low to allow it. Direct comparison of experimental data (Fleißner *et al.*, 2009b; Wright, G.D. and Read, N.D, unpubl. results) with our mathematical model suggests that the activator might be the active phosphorylated form of the MAP kinase, MAK-2 (Goryachev *et al.*, in press; Fig. 5). The identity of the inhibitor is less clear but the inhibitor may not even exist! Inhibition of the activator may be simply achieved by depletion of the substrate that is used up during the activator pulse and needs replenishing during the refractory period before the system can undergo another burst of activity. Because slow mechanisms based on gene transcription are likely not involved in the ping–pong mechanism (Read *et al.*, 2009; Goryachev *et al.*, in press), fast positive feedback loops will be necessary for rapid signal amplification and various potential positive feedback mechanisms that could convert the MAK-2 MAP kinase system into an excitable system have been suggested. Although the precise molecular function of the SO protein is unknown, our model suggests that it may be involved in the signal release phase associated with fusion of the chemoattractant containing vesicles with the apical plasma membrane of the CAT (Goryachev *et al.*, in press; Fig. 6).

It has been proposed that the ping–pong signalling mechanism may have evolved in filamentous fungi to increase the efficiency of fusion between non-motile cells. Sessile conidia or conidial germlings depend on being close enough to potential fusion partners in order to span the distance by CAT growth and fusion. The ability to switch back and forth between being a signal sender and receiver would allow any

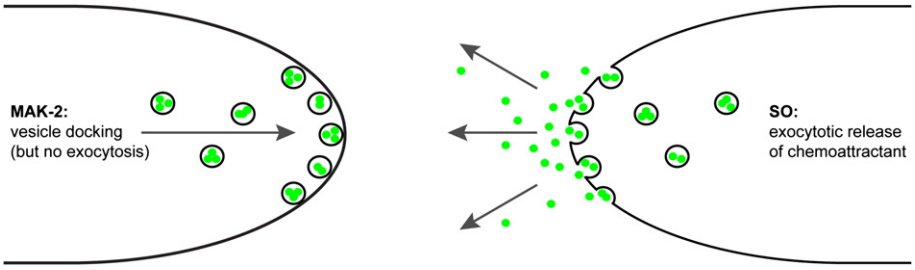

Fig. 6 – Simple model of how MAK-2 and SO may cooperate in regulating pulsatile secretion during CAT chemoattraction. It is proposed that phosphorylated MAK-2 promotes the docking of secretory vesicles, but inhibits their fusion, with the apical plasma membrane of a CAT homing towards another CAT. SO, on the other hand, may promote the fusion of these vesicles with the plasma membrane and thus the release of chemoattractant molecules (shown as green dots). It is predicted that only the pronounced transient accumulation of SO at the CAT tip will result in a burst of chemoattractant release.

fusion-competent cell to fuse with any other genetically identical, fusion-competent cell in its vicinity. This contrasts with genetically identical mammalian macrophages that can 'differentiate' into different physiological states and simply move towards, make contact and undergo self-fusion with a macrophage in an alternative physiological state (Read *et al.*, 2009).

7. Cell attachment and fusion pore formation

Once CATs make contact with each other they cease tip growth and firmly adhere to each other (Fig. 2e). The intervening cell wall is then remodelled and degraded, presumably by hydrolytic enzymes secreted from the CAT tips. This is followed by the plasma membranes of the two CAT tips merging and the development of a fusion pore which finally results in cytoplasmic continuity being achieved between the fused CATs (Fig. 2e–g). The PRM-1 protein is involved in plasma membrane merger (Fleißner *et al.*, 2009a) and three other proteins (HAM-6, HAM-7 and HAM-8) have been proposed to be also involved in these final stages of CAT fusion (Fu *et al.*, 2011). Other proteins that are localized at the fusion pore, and thus seem to play a role in fusion pore formation, are the MAP kinase MAK-2 (Fleißner *et al.*, 2009a), the polarisome proteins BNI-1, BUD-6 and SPA-2 (Lichius *et al.*, 2012) and F-actin (Berepiki *et al.*, 2010; Roca *et al.*, 2010; Lichius *et al.*, 2011). Once the fusion pore is established, cytoplasmic continuity between the anastomosed CATs is achieved and organelles can move between the fused partner cells (Ishikawa *et al.*, 2010, 2012; Roca *et al.*, 2010; Ruiz-Roldán *et al.*, 2010).

8. Other signalling pathways and proteins involved in CAT fusion

From mutant analyses in *N. crassa* (Table 1), it is clear that a number of other signalling pathways and proteins are involved at different stages of CAT fusion. These include:

(1) *The STRIPAK complex* (HAM-2, HAM-3, HAM-4, MOB-3, PP2A). Deletion mutants of several components of the striatin-interacting phosphatase and kinase (STRIPAK) complex (Goudreault *et al.*, 2009) are defective in CAT formation. The HAM-2 protein is a transmembrane protein that is a homologue of Far11p in the budding yeast (Xiang *et al.*, 2002) and PRO22 in *Sordaria macrospora* (Bloemendal *et al.*, 2010). In *S. macrospora*, which is closely related to *N. crassa*, PRO22 is predominately localized in the tubular and vesicular vacuolar network of vegetative hyphae. HAM-3 is a homologue of budding yeast Far8p, which is a calmodulin-binding, striatin protein and HAM-4 is a homologue of Far9p/10p, which is a forkhead associated protein (Simonin *et al.*, 2010). MOB-3 is a homologue of mammalian phocein that interacts with striatin that has been suggested to act as a scaffolding protein linking cell signalling and endocytosis (Benoist *et al.*, 2006). Far8p, Far9p, Far10p, Far11p are components of the Far11p complex in budding yeast that plays a role in regulating pheromone stimulated G_1 cell cycle arrest (Kemp and Sprague, 2003). PP2A is the catalytic subunit of protein phosphatase A, a serine/threonine phosphatase with broad specificity and diverse cellular functions (Milward *et al.*, 1999). It is presently unclear what role the STRIPAK complex plays in vegetative cell fusion in *N. crassa*.

(2) *Endocytosis*. The protein AMPH-1 is an amphyphisin protein and homologue of RVS161 and RVS167 in budding yeast. Amphyphisins contribute to endocytosis by inducing plasma membrane curvature (Berepiki *et al.*, 2011). The *Δamph-1* mutant is unable to undergo CAT formation. Actin patches are generally regarded as sites of endocytosis and are abundant in CATs at different stages of CAT fusion (Berepiki *et al.*, 2010; Lichius *et al.*, 2011). Amongst other functions, endocytosis is important for polarized growth and for recycling membrane proteins (Peñalva, 2010), processes that are undoubtedly of great importance at different stages of CAT fusion. As indicated above, a possible link between signalling and endocytosis, involving the STRIPAK complex, may occur (Maerz *et al.*, 2009).

(3) *The cell wall integrity MAP kinase pathway*. The MAP kinase kinase kinase MIK-1, the MAP kinase kinase MEK-1 and the MAP kinase MAK-1 are all involved in the CAT fusion because deletion mutants of genes encoding the proteins are all defective in CAT formation (Lichius, 2010; Fu *et al.*, 2011; Table 1). A role for the cell wall integrity MAP kinase pathway (Qi and Elion, 2005) in cell fusion is to be expected given the significant amount of cell wall remodelling that

must occur during CAT formation and during the final stages of CAT fusion.

(4) *Redox signalling*. NOX-1 (NADPH oxidase), NOR-1 (NOX regulator) and RAC-1 (Rho GTPase) are key components of the NOX complex involved in redox signalling, and deletion mutants of genes encoding these proteins are defective in CAT induction (Δ*rac-1*) or CAT chemotropism (Δ*nox-1* and Δ*nor-1*). In the plant pathogen *Botrytis cinerea*, knockout mutants lacking the NADPH oxidase (*Bc*NoxA) and its potential regulator *Bc*NoxR were also inhibited in CAT fusion. Furthermore, CAT fusion was inhibited by the NOX inhibitor DPI and by the reactive oxygen species scavenger ascorbic acid (Lichius, 2010; Roca *et al.*, in press). The precise role that redox signalling plays in the process of CAT fusion is unclear but results obtained with *N. crassa* suggest that it is important during CAT chemotropism (Lichius, 2010).

(5) *Transcription factors* (ADA-3, ADV-1, PP-1, RCM-1, RCO-1 and SNF-5). ADV-1 is homologous to Pro1 which is a major transcription factor involved in regulating sexual development in *Sordaria macrospora* (Masloff *et al.*, 1999). PP-1 is the homologue of the budding yeast Ste12p that is activated by the MAP kinases in the yeast pheromone response pathway (Qi and Elion, 2005). RCM-1 and RCO-1 are the *N. crassa* homologues of the budding yeast Ssn6 and Tup1 proteins, which form a heterodimer that functions in regulating yeast cell growth (Aldabbous *et al.*, 2010). Transcriptional control of gene expression will undoubtedly be involved during CAT induction and during the final stages of CAT fusion.

9. Conclusions

Great advances have been made in understanding the mechanistic basis of self-fusion between conidial anastomosis tubes since they were first described in *N. crassa* 7 y ago (Roca *et al.*, 2005a). However, discoveries about this process that have been made since then have highlighted how much still remains to be elucidated. Key aspects that need attention include the identification of: the self-signalling ligand(s), the self-signalling receptor(s), regulatory components of the excitable ping–pong mechanism, and the regulatory networks involved in the different stages of CAT fusion. Of particular significance will be understanding how the sensing of the self-signalling ligand(s) is integrated with the entire network of signalling pathways and proteins that have been identified as well as those that remain to be identified. Another interesting question relates to the evolution of self-signalling and self-fusion mechanisms within the fungi as a whole.

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