Functional genomics and signalling in invasive growth of *Schizosaccharomyces pombe*

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Declaration

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another university for the award of any other degree.

Signature.....

Date.....

Acknowledgments

Firstly I would like to thank my supervisor Dr. John Armstrong for giving me this opportunity as well as his time, patience and expertise over the last four years. I also owe a great deal to former members of the laboratory: James Dodgson, Gill Owen and the late Neil Bone, who not only helped me with problems but additionally provided a friendly working environment. I am also grateful to Dr. Roger Phillips for his advice and technical expertise on microscopy.

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Summary

Schizosaccharomyces pombe is a unicellular organism which has been shown to undergo invasive filamentous growth under nitrogen deprived conditions. Through the completion of a genome-wide screen of *S. pombe* deletion mutants this invasive growth form has been separated into three stages: adhesion, invasion, filament formation. The filament formation of each deletion strain was analysed resulting in the classification of mutants into four morphologically aberrant groups: (Ia) strains that were unable to form filamentous protrusions, (Ib) strains that had thickened rope structures of their filamentous protrusions, (IIa) strains that had elongated cells in their filaments and (IIb) strains that had erratic structure of cells in their filaments. The screen also identified strains that exhibited altered levels of invasion efficiency. These were classified as hypo-invasive, poorly-invasive or hyper-invasive. Class Ia strains were selected for further analysis as they represent the tertiary stage of invasion, filament formation. To attempt to identify the signalling pathways involved in this stage, potential signalling compounds were added to growth media and any alteration in phenotypes were noted. cAMP, iron and calcineurin were all analysed for their roles in the tertiary stage of invasion. The non-invasive and poorly-invasive strains were also tested with these signalling compounds to attempt to elucidate their role in the secondary stage of invasive growth. Finally the role of spindle pole bodies (SPB) was analysed during filament formation. The SPB duplicates in late G1/S phase and in single cells the new SPB migrates to either cell end (new or old) in an un-biased pattern. Using GFP tagged SPB markers, the segregation pattern in filaments was analysed followed by creation of deletion mutant/SPB-GFP-tagged hybrids to attempt to elucidate the control of SPB segregation in filaments.

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Abbreviations:

5-FOA – 5 Fluoroorotic acid

BP –band pass

Br-cAMP – Bromo-cyclic adenosine mono-phosphate

BSA – bovine serum albumin

cAMP – cyclic adenosine mono-phosphate

CDS – protein coding sequence

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

ER - endoplasmic reticulum

G2/M –the growth phase 2/mitosis boundary of the cell cycle

GAP – GTPase activating protein

GEF- Guanine nucleotide exchange factor

GFP – green fluorescent protein

GO – gene ontology

LNB – low nitrogen base media

MAPK – mitogen activated protein kinase

mRNA – messenger ribonucleic acid

MTOC – microtubule organising centre

NEB- New England Biolabs

ORF – open reading frame

P-5-P – pyridoxal-5-phosphate

PCR – polymerase chain reaction

PE- Phosphatidylethanolamine

PI-3-K - phosphatidylinositol-3 kinase

PI-3-P - phosphoinositide-3-phosphate

PKA – protein kinase A

PN – pyridoxine

PP2A - protein phosphatase 2A

RNA – ribonucleic acid

SA – stress-activated

SIN – septation initiation network

SPB – spindle pole body

TOR – target of rapamycin

YES – yeast extract plus supplements

Chapter 1: An introduction to *S. pombe* and dimorphic fungi

1.1: Fungi exhibit dimorphic growth

Organisms that belong to the eukaryotic kingdom of fungi can be capable of unicellular or multicellular growth (Madhani et al. 1998). These organisms are dimorphic. *S. pombe* is a unicellular organism that can be induced to form multicellular, invasive structures (Amoah-Buahin et al. 2005). The formation of invasive structures is seen in many other fungi including *Saccharomyces cerevisiae (S. cerevisiae), Schizosaccharomyces japonicus (S. japonicus)* and *Candida albicans (C. albicans)* (Odds 1985; Gimeno et al. 1992; Sipiczki et al. 1998). The invasive structures constitute a central colony of cells that have filamentous protrusions radiating out from them. The transition to invasive growth involves changes to growth pattern and the cells display a different morphology to their yeast phase counterparts. This can be induced by poor environmental conditions and is thought to be a foraging response to search for nutrients (Madhani et al. 1998).

As *S. pombe* is a non-pathogenic model organism with a plethora of information and diagnostic tools available for its investigation, it is an excellent model system in which to study this transition. Here *S. pombe* is used to investigate the signalling and mechanistic changes that take place in the transition from unicellular to multi-cellular growth.

1.2: *S. pombe* is a fission yeast

1.2.1: The original description of *S. pombe*

S. pombe was isolated in 1893 by P. Lindner who described a novel ascus-forming yeast that was able to replicate by medial fission. *S. pombe* was described as cylindrical in shape and replication occurred by formation of a transverse wall that cleaves and separates the cell (Lindner 1893).

The name *Schizosaccharomyces* was chosen to illustrate its difference (form of division) yet similarities (formation of ascus) to the Saccharomycotina sub-phylum. The suffix *pombe,* which translates as beer in Swahili, was chosen as a reference to the East African millet beer from which the yeast was isolated. P. Lindner's original description is of a heterogeneous species that readily sporulates and is capable of branching and pseudo-hyphal growth under anaerobic conditions (Lindner 1893). This growth form is not so readily seen in 21st century laboratory stocks, as it has been bred out by selective pressure. However, its presence in the original description underlines the filament-

forming ability of this yeast. The isolation of this yeast provided a new model system in which to study a variety of processes.

1.2.2: Phylogenetic position

*S. pomb*e belongs to the genus *Schizosaccharomyces* in the sub-phylum Traphinomycotina which is in the phylum Ascomycota. Here it sits alongside three other yeasts: *S. japonicus*, *S. octosporus* and *S. cryophilus* (Rhind et al. 2011). These are characterised by their ability to form asci and replicate by medial fission. Through alignment experiments utilising eukaryotic orthologous groups (KOGs)(Kuramae et al. 2006)(Kuramae et al. 2006) *S. pombe* has been positioned basal to Hemiascomycetes (*S.cerevisiae*) (Kuramae et al. 2006).

1. 3: Single-celled growth of *S. pombe*

S. pombe is predominantly haploid and cells are typically 7-14μm in length and 4μm in diameter which results in a rod-shaped cell (Mitchison et al. 1985). These replicate roughly every 2 hours by medial fission. *S. pombe* is a fermenting yeast that can utilise variable fermentable and non-fermentable carbon sources, including glucose, glycerol and sucrose.

1.3.1: Life cycle

Single cell growth predominates at an optimal temperature of 30ºC until a nutritional source is depleted or the environment becomes inhospitable. Single cells of opposite mating type can then mate with one another to form a diploid which then normally sporulates immediately to produce spores which are highly resilient to harsh environmental conditions. These then lie dormant until favourable conditions return, at which point the spores germinate and cells return to a life cycle of growth and division (figure 1.1). *S. pombe* cells are only capable of mating in the presence of an appropriate mating partner. When possible *S. pombe* will conjugate then undergo karyogamy to form a zygote followed by meiosis, after which sporulation initiates at the spindle pole bodies (SPBs) to produce an ascus which will break down to release four haploid spores. During sporulation the four SPBs serve as a docking sites for the formation of the forespore membrane which eventually encapsulates the four haploid nuclei (Shimoda 2004). This process is thought to involve targeting of vesicles to the SPB which then fuse and act as a docking site for membrane growth (Shimoda 2004).

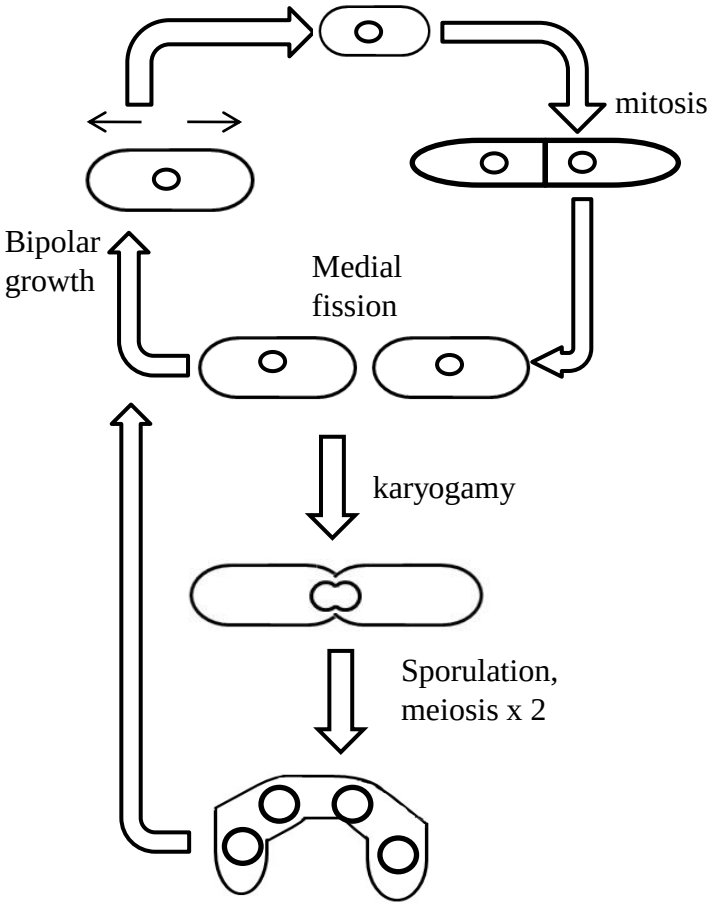


Figure 1.1 Life cycle of *S. pombe*. *S.pombe* grows by bipolar cell extension and divides by medial fission. Under poor nutrient conditions *S. pombe* will mate with a cell of opposite mating type and form haploid spores. These can sustain extremely harsh conditions until a favourable environment returns, at which point the spores germinate and return to a haploid life cycle of growth and division.

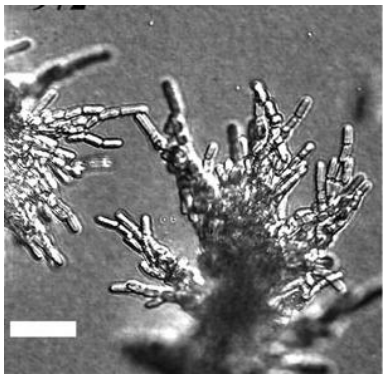


Figure 1.2 Invasive colonies formed by *S. pombe* . Bar 40µm. From Dodgson *et al*. 2009.

S. pombe is predominantly haploid though occasional diploids form. These are highly unstable and generally undergo spontaneous meiosis resulting in four haploid cells. This single cellular reproductive pattern was considered a complete representation of the *S. pombe* life cycle until 2005 when Amoah-Buahin *et al.* described a further low nitrogen-inducible phase of growth that was multi-cellular (figure 1.2). This newly described growth phase was reminiscent of invasive hyphal growth of pathogenic organisms such as *C. albicans*. The discovery of *S. pombe* as a dimorphic yeast provided an excellent model organism for the study of multi-cellular growth. Invasive growth is stimulated on low nitrogen, high carbon media (Amoah-Buahin et al. 2005). Nitrogen deprivation is also a stimulant of the mating response.

1.3.2: Mating

Nitrogen deprivation can cause G1 arrest followed by mating and production of haploid spores (Klar 2007). This can only occur between cells of opposite mating type as it requires activation by opposing pheromones. *S. pombe* has two mating types plus (h+) and minus (h-). Upon nitrogen deprivation cells secrete pheromones and produce pheromone cell surface receptors for the opposite pheromone (Klar 2007). H+ mating type cells release P pheromone and M pheromone receptor, Mam2, whereas h- mating type cells release M pheromone and P receptor, Map3. The cell surface receptors specifically bind the opposing pheromone of the host cell which initiates the sexual reproduction signalling cascade resulting in conjugation, karyogamy meiosis and subsequent sporulation (Klar 2007).

1.3.2i: G protein coupled receptors transduce the pheromone signal

Mam2 and Map3 are G protein coupled receptors (Kitamura et al. 1991; Tanaka et al. 1993). P or M pheromone binding causes a conformational change which results in Gpa1, α subunit disassociation and subsequent activation of the Spk1, Byr1, Byr2 mating MAPK signalling cascade (Obara et al. 1991; Xu et al. 1994). This signal transduction results in activation of Ste11, which is the master transcription factor in control of the mating response (Sugimoto et al. 1991). Ste11 up-regulates transcription of many target genes via binding to a consensus sequence (AACAAAGAAA) within their promoters (Mata et al. 2006). Activation of the mating response by pheromones

also leads to a reduction in intracellular cAMP. This is achieved through Ste11 which up-regulates expression of Cgs2, the phosphodiesterase responsible for degradation of cAMP (Mochizuki et al. 1992).

1.3.2ii: *S. pombe* strains can be homothallic or heterothallic

S. pombe cells can exhibit a homothallic or heterothallic life cycle (Gutz et al. 1973), which is determined by their ability to switch the mating type during DNA replication (Klar et al. 1991). Homothallic strains can switch their mating type locus. Therefore within a single colony of homothallic cells both P and M mating types will be present thereby facilitating mating within a single colony. Homothallic strains are also described as h^{90} as 90% of the population switch mating type (Klar 2007). In contrast, heterothallic strains are unable to switch mating type. Therefore cells within a single colony cannot mate and mating requires the addition of cells of opposite mating type.

1.3.2iii: Mat1 is the mating type locus

The mating type locus, Mat1 is located on chromosome 2 (Xiang et al. 2000). Mat1 contains either the P or M mating type allele, which are additionally stored and genetically silenced at Mat2 and Mat3 loci located 17kbp and 27 kbp centrosome distal to Mat1 respectively (Klar 2007). The allele, P or M, that is present at Mat1 determines the mating type of the cell (Beach et al. 1984). Homothallic cells are capable of switching this allele for the opposite allele from Mat2 or Mat3 during DNA replication (Beach et al. 1984).

1.3.2iv: Mating type switching does not occur every generation

Homothallic cells do not switch mating type every DNA replication cycle. Instead one in every four cells of a granddaughter population have switched mating type (figure 1.3) (Klar 2007). As one in every four granddaughters instead of one in every two daughters has a switched mating type, it implies it is a process that takes two cell replications to mature. Additionally, the imprinting must only affect one strand of DNA during replication, as if both strands were affected then two of four granddaughters would exhibit the switched phenotype (Klar 2007) (figure 1.3). This asymmetry in inheritance of switch-ability is proposed to be reliant on the lagging strand machinery imprinting a single DNA strand during replication (Dalgaard et al. 1999). To ensure Mat1 is replicated in a single direction, so that only one strand is imprinted by the lagging strand machinery, there is a polar replication termination sequence (RTS)

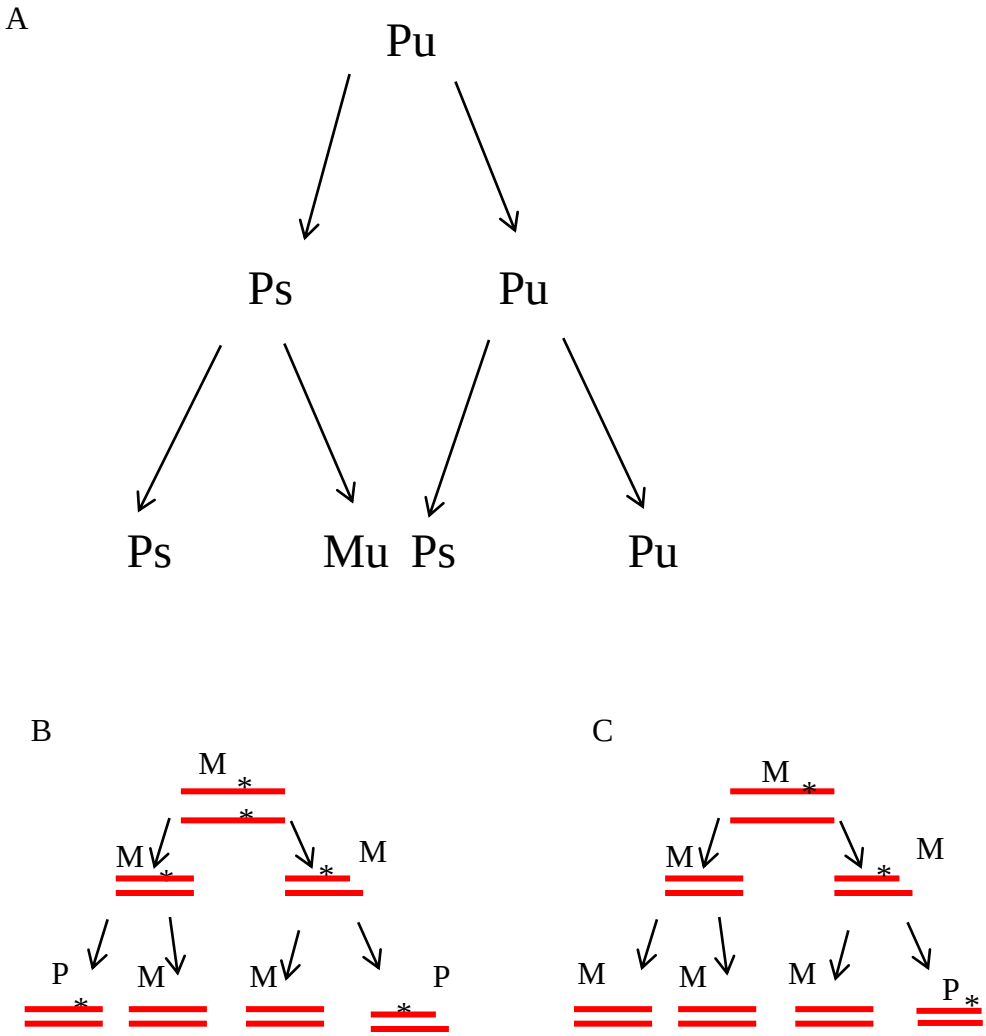


Figure 1.3. Mating type switching in *S. pombe.* A) S: switchable mating type, U: un-switchable mating type. 1 in every 4 granddaughters of an un-switchable parent is capable of switching mating type. A un-switchable parent produces a daughter that has the potential to switch and a daughter that does not. In the following cell cycle the daughter which inherited the potential to switch, switches mating type. B) If the DNA imprint (*) that allows cells to switch mating type affected both strands of DNA then 2 in every 4 granddaughters would switch mating type. C) If the DNA imprint (*) only affected 1 DNA strand then only 1 cell in a granddaughter population would switch mating type (Klar 2007).

2.5Kbp left of Mat1. Swi7 encodes the lagging strand DNA polymerase α and the deletion of *swi7* confers a loss of homothallic phenotype thereby supporting this theory (Dalgaard et al. 2001).

1.3.2v: Mating type switching involves a "nick" in the DNA

Within a homothallic colony cells are designated as switchable or un-switchable, this refers to the ability to interchange Mat1 during the following DNA replication (Gutz et al. 1973). The ability to switch mating type is conferred by an imprinting of DNA at the Mat1 locus (Arcangioli 1998). This imprint is thought to be a "nick" or RNA nucleotide replacement in the mat1 DNA. An un-switchable parent does not have this imprint, however upon DNA replication a single strand of its DNA acquires this nick (Arcangioli 1998). Therefore, upon cell division it produces one "nicked", switchable daughter and one "un-nicked", un-switchable daughter. The imprint does not manifest itself as an exchanged allele until the following DNA replication therefore one in every four granddaughters of an un-switchable parent switches mating type (figure 1.3). Once a cell has switched mating type, it is un-switchable in the following cell replication (Arcangioli 2000).

1.3.2vi: Mating and invasion are both stimulated by low nitrogen

Invasive growth and mating can both be considered responses to unfavourable conditions and the activation of both by low nitrogen suggests comparisons to the mating response may be helpful when considering invasive pathways. Spore formation enables *S. pombe* to survive in extremely harsh conditions, when favourable conditions return the spores germinate and revert to the previously described life cycle of growth and division which is under the control of the cell cycle. Whereas invasive growth can be considered a foraging response to search for better nutrient conditions.

1.3.3: The *S. pombe* cell cycle

S. pombe replicate roughly every two hours and their cell cycle consists of a long G2, followed by mitosis (M phase), and short G1 and S phase. During G1 the cell undergoes much protein synthesis to prepare the cell for the subsequent cell cycle. This is followed by S phase, where the DNA replicates. During G2 cells grow to roughly 14μm in length which is followed by growth arrest at 0.75 way through a cell cycle and mitosis, which separates the genetic content of the cell (Mitchison et al. 1985). Finally cells undergo cytokinesis by medial fission to produce two daughters cells of equal size. The control

of progression into these cell cycle phases is regulated by one master cyclin dependent kinase (Cdk) Cdc2.(Moreno et al. 1989; Fisher et al. 1996; Martin-Castellanos et al. 1996)

1.3.3i: Cdc2 is the main Cdk in control of the *S. pombe* cell cycle

Cdc2 levels remain constant throughout the cell cycle so its activity is governed by changes in cyclin availability (Nurse et al. 1980). This causes specific activation of different stages of the cell cycle. There are four main cyclins (Cig1, Cig2, Puc1 and Cdc13) that associate with and dictate activity of Cdc2 throughout the *S. pombe* cell cycle (Moser et al. 2000). Cig2 is the major cyclin in control of S phase (Fisher et al. 1996). Levels of Cig2 rise in late G1 and disappear after S phase (Martin-Castellanos et al. 1996). Cig2-Cdc2 complex is held inactive prior to S phase by the action of Rum1 kinase. On the progression to S phase, Rum1 is inhibited, potentially by Cig1-Cdc2 and Puc1-Cdc2 complexes, which alleviates inhibition of Cig2-Cdc2 and facilitates S phase entry (Martin-Castellanos et al. 1996). Cdc13 is the major cyclin required for Cdc2 activity during M phase (Moreno et al. 1989). Cdc13-Cdc2 remains inactive during S phase due to phosphorylation of Cdc2 on Tyr15 by Wee1 and Mik1 (Fleig et al. 1991). Upon transition to M phase, this inhibitory phosphorylation of Cdc2 is removed, in part by inhibition of Wee1 and Mik1 activity by Nim/Cdr1 and in part by activation of Cdc25, a phosphatase that removes the inhibiting phosphorylation on Cdc2 (Moreno et al. 1990; Parker et al. 1993).

The activity of these cyclins and therefore regulation of the cell cycle are subject to numerous forms of control including transcriptional, spatial and post translational. Dodgson *et al*. (2010) described *S. pombe* filaments as predominantly exhibiting G2 phase growth with elongated cells, suggesting there is control of the cell cycle involved during invasive growth. Therefore, elucidating this control mechanism could prove pivotal in understanding the invasive response.

1.3.4: Bipolar growth of *S. pombe*

S. pombe cells exhibit bipolar growth which is achieved by extension of the cell tips (Mitchison et al. 1985). Directly after cytokinesis growth is restricted to the old cell tip that was present prior to the previous division. Once a critical size of 9-9.5μm has been reached and cells have passed a specific point early in G2, (Mitchison et al. 1985), growth also occurs at the new tip formed from the previous cytokinesis. This

phenomenon is called new end take off (NETO). NETO is one of two changes in the localisation of growth machinery. The second is the switch to cell growth at the division septum during cytokinesis.

1.3.4i: Microtubules are involved in bipolar growth

Bipolar growth requires strict control of growth machinery to determine the site of cell growth. Microtubules are involved in this determination as well as in activation of actin polymerisation which is required for the physical extension of the cell.

The microtubule cytoskeleton is made of 3-5 anti-parallel microtubule bundles that radiate out from the nuclear envelope to the cell ends (Tran et al. 2001) (figure 1.4). Microtubules originate from microtubule organising centres (MTOCs) which are embedded within, or in close proximity to, the nuclear envelope (Hagan 1998). *S. pombe* has three different categories of MTOC that serve variable roles throughout the cell cycle. The interphase (i)MTOC serves to produce tracts for cellular transport as well as maintain the position of the nucleus centrally within the cell. The spindle pole body (SPB) is the mitotic MTOC and nucleates the mitotic spindle as well as astral microtubules, which serve to establish spindle orientation during mitosis. Finally the equatorial (e)MTOC is responsible for the spindle associated with the division septum and cellular cleavage (Heitz et al. 2001). The iMTOC along with auxiliary protein Ase1, organises the microtubules into anti-parallel bundles that overlap at their minus ends. iMTOC contains γ tubulin ring complexes that nucleate the microtubules out towards the cell ends (Loiodice et al. 2005). Mto1 and Mto2 recruit the γ tubulin ring complexes to the iMTOC thus facilitating polymerization of the microtubules by addition of α and β tubulin heterodimers (Samejima et al. 2005). Microtubules are required for positioning of cell growth components as well as maintenance of the central position of the nucleus in the cell (Tran et al. 2001).

1.3.4ii: Microtubules are dynamic structures

Microtubules are rigid structures which push against the cell end, and as the iMTOCs are associated with the nuclear envelope contact of the microtubules exerts a centralising force on the nucleus (figure 1.4) (Tran et al. 2001).

The microtubules are dynamic structures that grow towards the cell ends. These microtubules then act as tracts for delivery of growth components (Martin 2009). Once

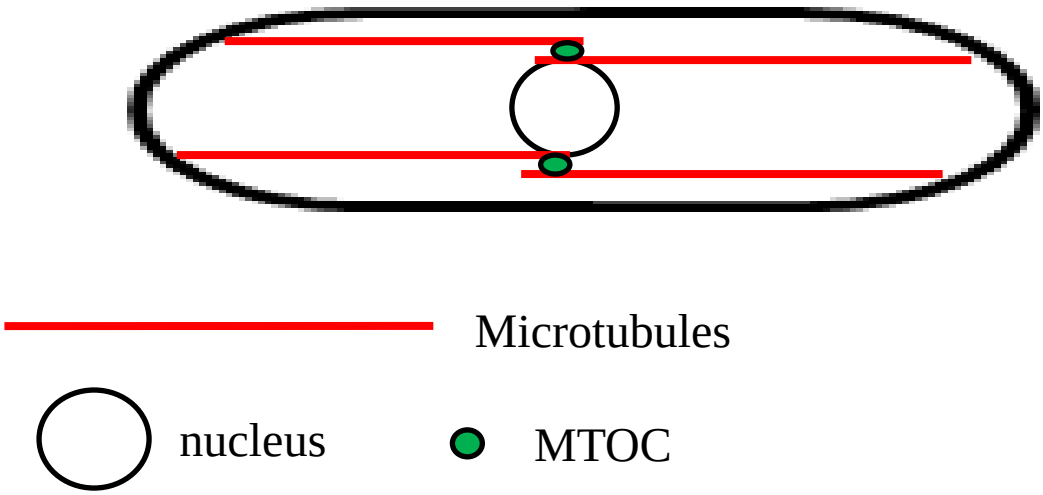


Figure 1.4 Microtubules are arranged in anti-parallel bundles that radiate out from the MTOCs to the cell tips. The force of the microtubules pushing on the cell ends centralises the nucleus (Tran *et al*. 2001).

the microtubules make contact with the cell end, they undergo microtubule catastrophe which involves depolymerisation and shrinkage (Drummond et al. 2000).

1.3.4iii: Delivery of growth components to the cell ends requires microtubule associated proteins

Tip1 and Mal3 associate to the growing end of the microtubules and stabilises them to ensure they do not undergo catastrophe until they reach the cell end and allow the delivery of growth components to the correct position (Busch et al. 2004). The association of microtubule plus ends with the cell end facilitates delivery of Tea1 and Tea4 (Martin 2009). These are transported along the microtubules as cargo of Tea2, a microtubule motor protein (figure 1.5). Tea2 is loaded onto the microtubules in a Mal3 dependent manner and "walks" along the microtubule in 8nm steps (Svoboda et al. 1993) via its intrinsic globular motor domains located at the N terminus of the protein (Browning et al. 2003). These exchange ADP for ATP resulting in a conformational change which manifests itself as a movement along the microtubule (Browning et al. 2000). Once delivered to the cell ends, Tea1 and Tea4 dock to Mod5 and Tea3 which are at the cell cortex. Tea1 is then required to dock Tea2 onto the cell membrane (Browning et al. 2003). Additionally Tea1 is required for restriction of microtubule extension at the cell ends; in *tea1Δ* cells microtubules do not undergo catastrophe and curve as they reach the cell end. This suggests Tea1 functions as a negative regulator of microtubule growth (Ste´ phanie La Carbona1 2006). Microtubules remain associated with the cell cortex for roughly two minutes before they undergo catastrophe thus retracting from the cell end. This is thought to be dependent on the disassociation of Tip1 (Busch et al. 2004).

1.3.4iv: Tea1 and Tea4 recruitment to the cell end activates For3

The delivery of Tea1 and Tea4 to the cell ends allows recruitment of Bud6 and For3 (Feierbach et al. 2004). For3 is a formin responsible for actin nucleation (Feierbach et al. 2001). For3 is activated by Bud6 and Cdc42 (Martin et al. 2007) (figure 1.5). Once tethered to the cell cortex, Tea1 activates Pom1 kinase, which subsequently inhibits the localization of Rga4. Rga4 is the GTPase activating protein for Cdc42, therefore the inhibition of Rga4 localisation effectively activates Cdc42. Cdc42 then activates For3 resulting in actin nucleation and cable production which serve as tracts for growth associated vesicle delivery (Martin 2009). Cellular growth is facilitated by the delivery

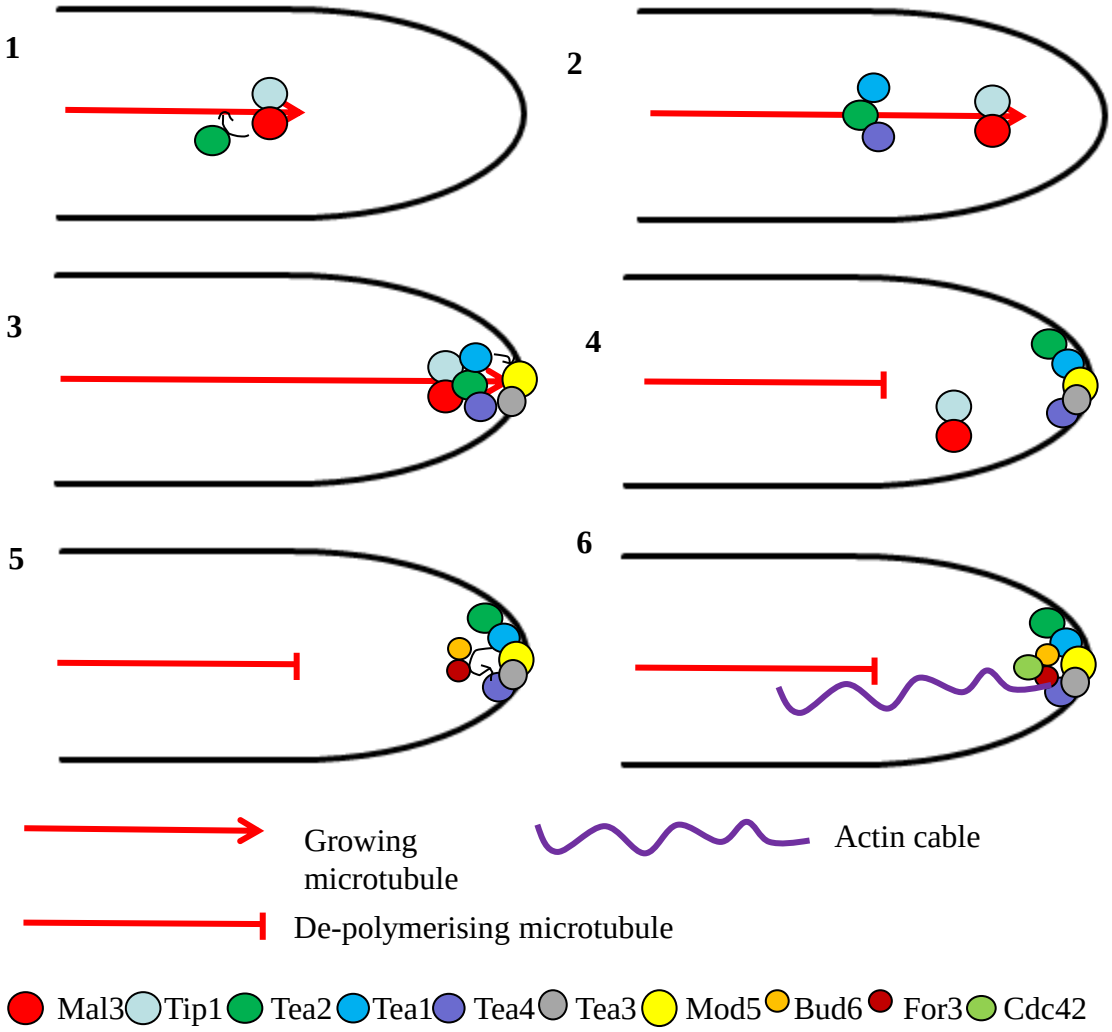


Figure 1.5 Microtubules facilitate delivery of cell polarity determinants to the cell end. 1) Growing microtubules are stabilised at the tip by Tip1 and Mal3. Mal3 recruits Tea2 to the microtubule. 2) Tea2 moves along the microtubule transporting cargo proteins Tea1 and Tea4. 3) Once the microtubule reaches the cell tip Tea1, Tea4 are tethered to the cell cortex by Mod5 and Tea3. Tea1 tethers Tea2 to the cell cortex. 4) Dissociation of Tip1 and Mal3 from the microtubules cause it to depolymerise and shrink away from the cell cortex. 5) Tea1 recruits Bud6 and For3 to the cell cortex. 6) Cdc42 localisation at the cell cortex activates For3 which nucleates actin cables.

of membrane vesicles to the cell tips, which fuse with the membrane thus increasing it in size.

Dodgson *et al.* (2009) described *for3Δ* deletion strain as non-invasive. Additionally, the phenotype of *mal3Δ, tea2Δ, tea1Δ, tip1Δ* and *tea4Δ* were described as morphologically aberrant, which highlights the importance of microtubule and actin facilitated polarised growth during invasion (Dodgson et al. 2009). The final site of cellular growth is the division septum which culminates in the separation of the two daughter cells. This signal to divide is provided by the septation initiation network.

1.3.5: The septation initiation network (SIN)

The cooperation between growth, mitosis and division is crucial for the viability of the cell and ensures division only takes place once mitosis is complete. This ensures each daughter cell inherits one set of DNA and an equal proportion of cytoplasmic material. This requires tight temporal and spatial organisation of cytokinesis. If a cell divides too early the DNA is not appropriately segregated between daughter cells; if division takes place too late then binucleate cells may occur. Spatial coordination ensures the division septum is formed in the correct place and the cell is equally partitioned. The septation initiation network (SIN) is in control of where cellular division takes place (Krapp et al. 2008).

1.3.5i: SIN is coordinated from the spindle pole bodies (SPBs)

SIN components coordinate septation from the spindle pole bodies (SPBs). The SPB duplicates during S phase, the process of which is not properly understood (Uzawa et al. 2004).

1.3.5ii: SIN is regulated by multiple proteins

SIN is only activated from one of the SPBs during anaphase to ensure the cell only goes through a single division (Krapp et al. 2008). The SIN activity of a SPB is determined by the guananine nucleotide bound state of Spg1 (Schmidt et al. 1997), a component of SIN machinery. The association of SIN components to the SPBs is facilitated by the scaffold proteins Sid4 and Cdc11 (Krapp et al. 2001) (figure 1.6). The N terminal domain of Cdc11 is the binding site for much of the SIN associated machinery. Sid4 tethers the Cdc11/Sid4 complex to the SPB via a core SPB component ppc89 (Tomlin et al. 2002).

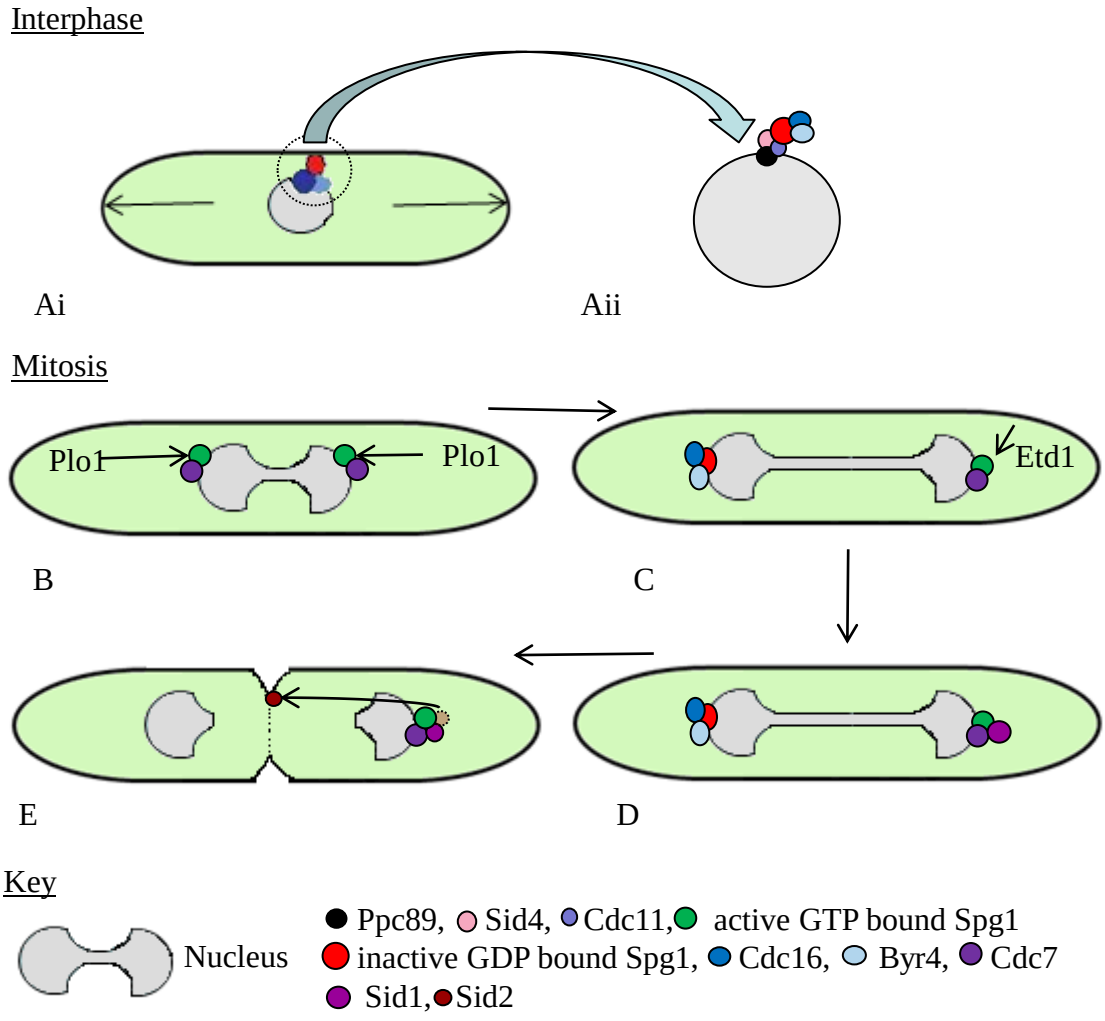


Figure 1.6 SIN is regulated from the spindle pole bodies (SPBs). Ai) During interphase SIN is repressed by Cdc16 and Byr4 presence on the SPB. The arrows within the cell represent bi-polar growth. Aii) Enlargement of nucleus and SPB to show the scaffold machinery. Sid4 and Cdc11 form the scaffold machinery to which the other SIN components attach. In all further depictions the scaffold machinery is not shown. B) As the SPBs start migrating to opposite poles Plo1 activates Spg1 on both SPBs. Active Spg1 recruits Cdc7. Arrows indicate activation. C) As the SPBs segregate further Spg1 remains active on one SPB, which is facilitated by Etd1 and is inactivated on the other SPB. This is facilitated by Cdc16/Byr4. D) Cdc7 on the active SPB then recruits Sid1. E) Sid1 then actives Sid2, which translocates to the contractile actin ring and actives septum cleavage.

SIN is predominantly a phospho-relay pathway that results in activation of cellular division (figure 1.6). The first kinase thought to function in SIN activation is Plo1, a polo like kinase which also functions during contractile actomyosin ring assembly (CAR). Plo1 is thought to activate Spg1 and function upstream of the main SIN components as activation of Spg1 in *plo1Δ* cells rescues its SIN mutant phenotype (Tanaka et al. 2001).

The activation of SIN is dependent on the guanine nucleotide bound state of Spg1 (Schmidt et al. 1997). Spg1 is a Ras family GTPase that can exist in active GTP bound state or an inactive GDP state (Krapp et al. 2008). In its GTP bound form, Spg1 recruits Cdc7 kinase to the SPB. This recruitment is essential for the transmission of the SIN signal. Cdc7 kinase then activates Sid1 kinase, which in turn activates Sid2 kinase causing its translocation to the CAR where it is thought to facilitate contraction (Guertin et al. 2000) (figure 1.6).

1.3.5iii: Spg1 activity is retained on the new SPB

In early mitosis Spg1 is active on both SPBs, therefore Cdc7 is recruited to both SPBs (Sohrmann et al. 1998). As the cell enters late anaphase Spg1, activity and therefore Cdc7 association, is lost on one SPB and retained on the other. Localisation experiments with Pcp1.dsRFP and Cdc7.GFP have shown that Cdc7 always localises to the new SPB in single cells and this new Cdc7 bound SPB is seen migrating with equal frequency to old and new end of the cell (Sohrmann et al. 1998; Grallert et al. 2004). Pcp1 is an SPB core protein which has been fused to dsRFP, a slow folding fluorophore. Pcp1.dsRFP takes a long time to fold so is only visible if cells are subjected to starvation, which forces them into G1 arrest where the red fluorophore has time to fold. Once the cells are re-fed and go through a subsequent cell cycle it is possible to distinguish the newly formed SPB from the old SPB (Grallert et al. 2004). Grallert *et al.* (2004) showed that Cdc7.GFP and Pcp1.RFP never co-localise, therefore Cdc7.GFP localises exclusively to the new SPB.

The activity of Spg1 is controlled by a de-activating “GTPase activating protein” (GAP) complex and activating protein Etd1 (Furge et al. 1998; Garcia-Cortes et al. 2009). Etd1 activates Spg1 but is not thought to be a guanine nucleotide exchange factor (GEF) that are normally responsibly for small GTPases activation (Garcia-Cortes et al. 2009). Cdc16/Byr4 is a GAP for Spg1; the phenotype of *cdc16Δ, byr4Δ* is reminiscent of Spg1 or Cdc7 over-expression with multi-septate cells and de-regulation of septum formation.

The Cdc16/Byr4 complex associates with the SPB throughout interphase and keeps Spg1 inactive. At the onset of mitosis Cdc16/Byr4 leaves both SPBs allowing partial activation of Spg1 (Furge et al. 1998). Full activation is not achieved until Cdc2/Cyclin13 activity is lost at anaphase. This full activation is only seen on the new SPB in single cells (Tanaka et al. 2001). Concomitant with full activation of Spg1 on the new SPB, Cdc16/By4 re-associates with the old SPB thus deactivating Spg1. This ensures SIN is only induced once. Fin1 is a NIMA kinase that associates with the SPB by binding directly to Byr4 (Grallert et al. 2004). Fin is involved in inhibition of SIN from the old SPB. However, Fin1 also associates to the new SPB via an unknown protein. The purpose of Fin1 association to the new SPB has yet to be defined. The recruitment of Fin1 to the SPBs in a cell is dependent on the history of the SPB prior to duplication. In some cells both the old and the new SPB will inherit Fin1, and in other cells only the old SPB will inherit it. Fin1 can associate to an old SPB and a new SPB but only if that new SPB has arisen from a duplication of a SPB that was old in the previous cell division (Grallert et al. 2004).

1.3.5iv: SIN causes contraction of the actomyosin ring

S. pombe divides by medial fission facilitated by a contractile actomyosin ring (CAR) (Bathe et al. 2010). The CAR forms throughout mitosis and contracts after its completion when triggered to do so by the septation initiation network (SIN). The site of CAR formation is determined early in mitosis when Mid1 is phosphorylated by the kinase Plo1. This causes its translocation from the nucleus to the equator of a cell where it forms a ring which is thought to act as a scaffold for the association of the future CAR components (Tanaka et al. 2001).

1.3.5v: Cellular checkpoints delay SIN to maintain cell integrity

To ensure SIN only progresses once DNA segregation has been completed efficiently there are checkpoints in place. One of these, the mitotic spindle check point, results in ubiquitination of Sid4 by Dma1 if the sister chromatids are not correctly associated with the mitotic spindle (Johnson et al. 2011). Sid4 is a scaffold protein that facilitates the association of SIN components to the SPB during SIN (Krapp et al. 2001). This ubiquitination of Sid4 results in the inability of Plo1 to associate with the SPB and so no SIN activation (Johnson et al. 2011). This functions to delay SIN until the sister chromatids are correctly associated with the mitotic spindle and able to separate. In this

model Johnson *et al*.(2011) propose that Plo1 association to the SPB in early mitosis is responsible for Byr4 phosphorylation and that this phosphorylation results in disassociation of Byr4/Cdc16 complex from the SPB, thus allowing Spg1 activation. Upon Sid4 ubiquitination, Plo1 is not recruited therefore Byr4 is not phosphorylated and remains at the SPBs keeping Spg1 in the inactive GDP bound state (Johnson et al. 2011). In support of this idea, Lahoz *et al*. 2010 found that a regulatory subunit of protein phosphatase 2A (PP2A), Pab1, functions antagonistically to Etd1, the Spg1 activator. Pab1 deletion partially rescues Etd1 SIN mutant phenotype. In *etd1Δ* cells the positive regulation of Spg1 is lost therefore it remains inactive at the SPB. When the proposed antagonistic phosphatase Pab1 is also deleted the inhibition is removed thus resulting in Spg1 activation. Lahoz *et al*. suggest this is oriented around the phosphorylation state of Cdc16/Byr4 complex. Pab1 has been shown to associate with SIN components Sid1 and Sid2 via two hybrid screens, which suggests it may be involved in SIN regulation at various levels (Lahoz et al. 2010).

The coordination of SIN is a complicated process and the machinery is subject to regulation from multiple cellular cues. Invasive growth involves cellular elongation and delaying SIN may facilitate this. Therefore the study of SIN during invasion may highlight proteins involved in its regulation.

1.4: Invasive growth

Invasive growth is a broad term used to describe the formation of multicellular structures that penetrate the growth medium. This growth form is exhibited by many ascomycota and the morphology of the cells within the multicellular structure defines the names used to describe them. Organisms such as *C. albicans* form true hyphae (figure 1.7) (Elson et al. 2009), which are extensively elongated cells with septa that allow cytoplasmic flow through them (Whiteway et al. 2007). Invasive growth of a yeast colony results in many hyphae forming and these are collectively called mycelium. Yeast cells are also capable of a transition to pseudohyphal growth. Pseudohyphae exhibit cellular elongation, though not as extreme as true hyphae. During pseudohyphal growth cells go through cytokinesis but they remain attached after this event. Additionally, pseudohyphal growth is associated with the formation of filaments on the surface of the growth medium rather than its penetration (figure 1.7) (Dickinson

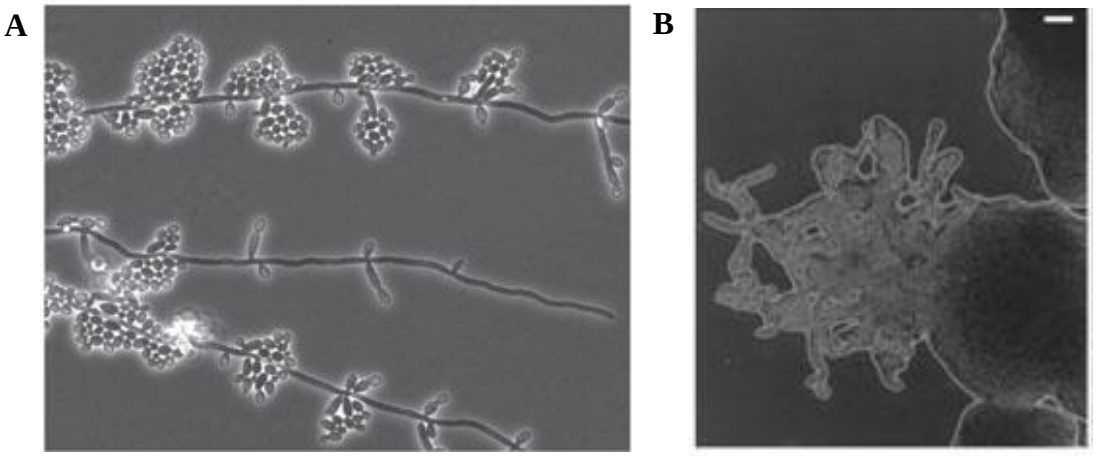


Figure 1.7 Different forms of filamentous growth. A) True hyphal growth of *C. albicans*. From Elson *et. al.* 2009. B) Pseudohyphal growth across the surface of the media. From Sipiczki *et al.* 1998.

2008). True hyphae and pseudohyphae are both forms of filaments. The multicellular structures formed by *S. pombe* do not align with the definition of either hypha or pseudohyphae, therefore the invasive structures formed by *S. pombe* will be referred to as filaments.

1.5: Invasive growth of *S. pombe*

S. pombe forms invasive, multicellular filamentous structures when grown on a low nitrogen, high carbon media (Amoah-Buahin et al. 2005). The trigger for the transition to invasive growth on this media is low nitrogen. Stimulation of invasion also requires a high cell density (Dodgson et al. 2009). This is suggested to involve a quorum sensing mechanism but the details of this remain elusive. The high cell densities range from liquid suspensions of 2 x 10^7 cells/ml (Mitsuzawa 2006) to large masses of cells (Dodgson et al. 2009). *Amt1Δ, amt2Δ* and *amt3Δ* were described as non-invasive (Mitsuzawa 2006). However further investigation showed that these genes were only required for invasion at the relatively low density of 2 x 10^7 cell suspension as they form invasive structures at the higher cell density (James Dodgson, unpublished data). Amt1, Amt2 and Amt3 are ammonium permeases. This suggests that at a high cell density the trigger for invasion is no longer restricted to stimulation by ammonia but perhaps invasion is then also stimulated by products of cell metabolism as has been suggested for *S. cerevisiae* (Dickinson 2008).

More recently high environmental iron has been shown to artificially activate invasive growth; treatment with iron quenching ferrizone results in a lack of invasion and addition of iron to growth medium results in a hyper-invasion (Prevorovsky et al. 2009). Following the original description *S. pombe* was also found to form invasive structures on nitrogen rich media, though this was less efficient than induction of invasion by low nitrogen (Dodgson et al. 2009). Subsequent research into *S. pombe* invasive growth has shown that the original strain in which the invasive phenomenon was discovered was, ironically, invasion-defective in comparison to other laboratory strains. Attempts to map the defective trait were unsuccessful and suggest an epigenetic inheritance system (Dodgson et al. 2009). Prior to invasion cells must first adhere to the agar. This adhesion is thought to be galactose dependent (Dodgson et al. 2009). Once cells have adhered they penetrate the media and form invasive structures. Invasive structures constitute a central focus of densely pack cells that have filamentous protrusions

radiating out from them. The filaments of these structures grow in a monopolar fashion. Replication of cells within these filaments takes roughly 2 hours and cells divide by medial fission (Dodgson et al. 2010).

1.5.1: Morphology of *S. pombe* invasive structures

Cells in the filaments are elongated, parallel sided and uniform in shape apart from the end cell which is more elongated in comparison to the others. Filament cells are mono-nucleate and appear to have vacuoles that vary in size in comparison to single cells. These vacuoles appear to be excluded from the growing tip of the filament cell. Filaments, like single cells, are thought to have a long G2 (Dodgson et al. 2010). Invasive cells are also capable of forming lenticular colonies (Dodgson et al. 2009). These are tightly packed structures of single cells. No filaments protrude out from the lenticular colonies.

1.5.2: Role of the cytoskeleton in invasive growth

The actin cytoskeleton plays a major role during invasive growth as treatment with Latrunculin A, an actin depolymerisation agent, inhibits invasion (Dodgson et al. 2009). Additionally, Pohlmnn *et al*.(2010) document that cold sensitive mutants of *act1, sop2, arp3* and a temperature sensitive mutant of *cdc8* are non-invasive at permissive temperatures. These proteins all have roles in actin patch or filament assembly and organisation therefore the inability of these strains to form invasive structures implicates the actin cytoskeleton in invasion (Pohlmann et al. 2010). The role of the interphase microtubule cytoskeleton is less important in invasive growth. Deletion strains representing microtubule associated polarity proteins (*tea1, tea2, mal3* and *tip1*) form invasive structures, however these exhibit morphological aberrations (Dodgson et al. 2009).

The role of the cytoskeleton in invasive growth was further investigated by GFP localisation within growing filaments.

1.5.3: Localisation of cytoskeleton components in filaments

A variety of proteins associated with different aspects of cellular growth exhibit asymmetric localisation in the leading cell of invasive filaments (Dodgson et al. 2010). Bgs4 is involved in cell wall synthesis (Cortes et al. 2005), Crn1 is an actin patch marker (Humphries et al. 2002) and Cdc42-GTP is the activator of actin polarisation (Martin et al. 2007). Bgs4-GFP and Crn1-GFP both localised to the growing end of the

leading filament. Additionally, "Cdc42/Rac interaction binding" (CRIB) domain of *Sc.*GLC2 which only binds to active Cdc42-GTP also localised to the growing end of the filament. Surprisingly cell polarity determinants Tea1, Mod5, Pom1 and Tea4 all localise to the non-growing filament end (Dodgson et al. 2010). Tea1 is part of the +TIP complex that is responsible for growth site determination (Mata et al. 1997). Tea4 is responsible for correct Tea1 localisation (Tatebe et al. 2005). Mod5 tethers these to the plasma membrane and Pom1 has multiple functions during polarised growth (Bahler et al. 1998; Snaith et al. 2003). Further analysis of Tea1-GFP localisation showed that Tea1 is being delivered to the growing end of the filament but it does not appear to be retained there. The asymmetric localisation of these cell polarity determinants suggests they are not required for maintenance of monopolar growth once it has been established (Dodgson et al. 2010).

Bud6, which is a For3 activator (Martin et al. 2007), Rga4 which is a GAP for Cdc42 (Tatebe et al. 2008) and Mid1 all show bipolar localisation during filamentous growth. Interestingly For-3GFP could not be visualised in filaments as the strain was non-invasive. This underlines the importance of For3 during the invasive process as even fusions to its C terminus can affect the invasive phenotype (Dodgson et al. 2010).

1.5.4: Characterisation of a *S. pombe* strain that is highly efficient at invasion

Localisation of proteins within filaments was achieved by confocal microscopy of actively growing filaments and was facilitated by the discovery of a highly efficient *S. pombe* isolate, strain A1153. Strain A1153 forms filaments that extend beyond the mass of surface cells that cause invasion; a trait that is not exhibited by the common laboratory strain 972 (Dodgson et al. 2010). This phenotype was exploited for live cell imaging to analyse the localisation of proteins within actively growing filaments.

1.5.5: Signalling events during invasion

Invasion does not require the pheromone activated, Spk1, mating MAPK cascade nor the stress-activated Sty1 or cell-integrity Pek1 MAPK cascades (Amoah-Buahin et al. 2005). However the cAMP/PKA signalling pathway is crucial as deletion of key components in the transduction cascade (*git3*, *git11* and *cyr1*) resulted in a non-invasive phenotype (Amoah-Buahin et al. 2005). Asp1 has been implicated as an essential component of invasive growth of S. pombe {Pohlmann, 2010 #189). Asp1 belongs to

the Vip1 family kinase and produces an IP7 isomer and IP8 (Mulugu et al. 2007). These are inoisitol polyphosphates that have been suggested to act as intracellular secondary messengers (Draskovic et al. 2008). Asp1 is a dual domain protein with a kinase domain and a N terminal phosphatase like domain but no enzymatic activity has been described for this domain to date. Asp1 is thought to be a regulator of Arp2/3 complex which is involved in actin nucleation (Pohlmann et al. 2010). Through creation of kinase only and kinase dead domain variation of Asp1, Pohlmann *et al*. (2010), provide data that suggests the kinase domain is crucial for the switch to invasive growth. They go on to suggest this activity is negatively regulated by the phosphatase domain on the C terminus. The kinase domain could be responsible for production of an IP7 isomer or IP8 which can serve as signalling molecules within the cell. They outline Asp1 functioning downstream of cAMP in the PKA pathway (Pohlmann et al. 2010).
The signalling processes during invasive growth have been crudely studied and further research into this area would provide insight not only into the regulation of invasion but also general control of polarisation.

1.5.6: Deletion strains that are known to display aberrant filamentous phenotypes

Amoah-Buhin *et al*. were the first to describe *S. pombe* as forming invasive structures that had filamentous protrusions but it was not the first documentation of filament formation. Lkh1 has been implicated as negative regulator of filamentous growth (Kim et al. 2001). *lkh1* was predicted to be important in polarised growth due to its sequence alignment with *Sc.KNS1*, a *S.cerevisiae* gene that functions in filamentous growth. The *lkh1* deletion strain showed adhesive, pseudohyphal surface growth, but no invasion. Kim *et al.*(2001) showed that although Lkh1 is not essential for growth it appears to be a negative regulator of adhesion and filament formation; *lkh1Δ* binds to the agar surface under good nutrient conditions and forms pseudohyphal filaments (Kim et al. 2001). Additionally, Sep1 is involved in cell separation and deletion of this gene results in strings of connected cells that are unable to separate properly (Ribar et al. 1999).
S. pombe has also been reported to form filamentous chains which is stimulated by fusel alcohols (Dickinson 2008), though fusel alcohols do not appear to affect invasive growth of *S. pombe* (James Dodgson, unpublished data).

Tea1, Tea4, Mal3, Tea2 and Tip1 are cell polarity controlling proteins. Deletion of these genes results in invasive structures that have aberrant filament morphologies (Dodgson et al. 2009).

Further analysis of *tea1Δ* in a hyper-filamentous background showed that the cells exhibited impaired directionality resulting in growth of curved filaments (Dodgson et al. 2010).

The aberrant morphologies displayed by these deletion strains represent a new class of invasive mutant which has not yet been studied. Deletion strains that are able to invade the media but form aberrant filaments represent proteins involved in regulation of filament formation.

1.5.7: Deletion strains that are known to display aberrant invasive phenotypes

The Bioneer *S. pombe* deletion library was screened for strains which failed to invade (Dodgson et al. 2009). The Bioneer deletion library used in this screen was a collection of 2638 strains each with a specific gene deleted. This identified 12 strains that were non-invasive. Five of these strains were also unable to adhere to the media. This led to the description of the a primary stage of invasion as adhesion (Dodgson et al. 2009). Two of these proteins, subsequently named Adn2 and Adn3, are transcription factors that have a domain related to the LUFS (LUG/LUH Flo8, single stranded DNA binding protein) domain in Flo8 of *C. albicans*. This domain is essential for hyphal development and virulence (Cao et al. 2006). A further transcription factor, subsequently named Adn1, has weak homology to a transcription factor in *A. thaliana* that binds to the LUFS domain. The domain required for this binding is not repeated in the *S. pombe* Adn1 orthologue so any interaction with Adn2 and Adn3 may be indirect (Dodgson et al. 2009). The other strains that were unable to adhere to the media represent Tlg2, a SNARE membrane trafficking protein and Snf5 which is part of the SWI/SNF complex and is involved in chromatin remodelling.

Dodgson *et al*, 2009, also identified seven strains that were able to adhere to the media but unable to invade (table 1.1). For3, which is a formin responsible for actin cable assembly (Feierbach et al. 2001), is the only protein involved directly with cellular growth. The others have functions that range from chromatin remodelling (Arp42, Sgf73) to cellular protein destruction (Ubr1) (http://old.genedb.org/genedb/pombe/).

Phenotype	**Gene**	**Function**
No Invasion No adhesion	*SPAC2F7.08c* *snf5*	Chromatin remodelling complex
No invasion	*SPBC21.05c* *ral2*	Involved in ras1 activation
No invasion	*SPBC11B10.07c* *ivn1*	CDC50 domain protein, localizes to the endosome membrane (predicted).
No invasion	*SPAC3H8.10* *spo20*	Involved in ascopspore formation and cytokinesis
No invasion No adhesion	*SPBC30B4.03c* *adn1*	Transcription factor (predicted)
No invasion	*SPAC23D3.09* *arp42*	SWI/SNF and RSC complex subunit
No invasion	*SPBC19C7.02* *ubr1*	Ubiquitin-protein ligase (E3)
No invasion No adhesion	*SPAC823.05c* *tlg2*	SNARE, 1 predicted transmembrane helix
No invasion	*SPCC126.04c* *sfg73*	Involved in chromatin silencing
No invasion No adhesion	*SPBC1289.10c* *adn2*	Transcriptional regulator (predicted)
No invasion No adhesion	*SPCC1494.10* *adn3*	Transcriptional regulator (predicted
No Invasion	*SPCC188.07* *for3*	Formin involved in activation of actin filament bundle assembly

Table 1.1 Deletion strains from the Bioneer collection that are unable to invade or adhere on YES media (Dodgson et al. 2009). All gene descriptions are from http://old.genedb.org/genedb).

Fep1Δ cells also exhibit a non-invasive phenotype (Prevorovsky et al. 2009). *Fep1* encodes a negative regulator of iron-assimilation genes (Pelletier et al. 2002). The level of intracellular iron in *fep1Δ* is higher than the wild type control but its transcriptome resembles that of ferrizone treated cells. Ferrizone is an iron chelator which means iron cannot be taken up by the cells. As *fep1Δ* transcriptome resembles that of the ferrizone treated wild type control, despite the higher intracellular iron levels, it suggests *fep1Δ* may not be able to sense the intracellular iron (Prevorovsky et al. 2009). This implies iron could be involved in invasion.

1.5.7i: Fep1 inhibits transcription of iron assimilation genes

Cellular iron readily gains and looses an electron which makes it an important co-factor in many reactions. It is this same redox capability of iron that requires its tight cellular

control as too much iron will result in reaction with hydrogen peroxide to from damaging hydroxide radicals (Labbe et al. 2007). The tight control of iron homeostasis is achieved by Fep1, which is an iron binding transcriptional repressor. Fep1 transcriptionally represses iron uptake proteins in high iron conditions; this repression is dependent on association of Fep1 and iron. When iron is low, Fep1-iron association does not occur. This results in lack of Fep1 transcriptional repression due to its inability to bind DNA. Fep1 not only controls iron transport proteins but also transcription of genes whose products encode iron-associated and iron storage proteins (Pelletier et al. 2002). The transcriptional control of the latter set of genes is controlled via the Php complex. This is a 4 subunit complex, 3 of which (Php2, Php3 and Php5) are continuously associated with the target genes. The fourth subunit, Php4 is responsible for the regulation of gene expression. When Php4 is present, it inhibits transcription from these promoters, and when it is not bound, transcription takes place. Php4 is negatively regulated by Fep1, so when iron is low Fep1 does not inhibit Php4 expression therefore it is up-regulated. Increased Php4 levels results in its binding to the Php2, Php 3, Php 5 complex and inhibition of gene expression (Labbe et al. 2007).

1.5.7ii: The ammonium transporter family

The *amt* family of genes have also been shown to function in invasive growth (Mitsuzawa 2006). These were characterised due to homology with a similar family of genes in *S. cerevisiae*. The *S. cerevisiae MEP* genes are subject to nitrogen catabolite repression (Marini et al. 1994); when nitrogen levels are high the genes required for nitrogen utilization are repressed, and when nitrogen levels are low they are activated. Low nitrogen is a stimulant for invasive growth of *S. cerevisiae* (Gimeno et al. 1992) as well as *S. pombe* and as such the *MEP* genes of *S.cerevisiae* are involved in invasive growth. *S. cerevisiae* has three MEP genes, which encode ammonium transporters (Lorenz et al. 1998). The crystal structure of *E. coli* ammonium transporters have been determined and these consist of a trimer where each monomer has 11 transmembrane helices. These have a NH_4^+ binding site and a NH_3 channel (Khademi et al. 2004; Mitsuzawa 2006). MEP2 is a high affinity permease and is required for cAMP mediated stimulation of pseudohyphal growth when ammonium is the sole nitrogen source. When other nitrogen sources form the limiting supply, such as glutamine or proline, MEP2 is not required for stimulation of pseudohyphal growth. Therefore, MEP2 is considered an

ammonium sensor that activates signalling that results in pseudohyphal differentiation (Lorenz et al. 1998).

The MEP genes of *S. cerevisiae* were used in a BLAST homology search of the *S. pombe* genome. There are 3 *MEP* genes in *S. cerevisiae* and 3 orthologues were identified in the BLAST search; *amt1, amt2* and *amt3*. These were analysed for their role in low nitrogen-induced invasion. Deletion of *amt1* or *amt2* results in poorly-invasive phenotypes (Mitsuzawa 2006) however, no such phenotype was documented for the *amt3Δ*. The addition of exogenous cAMP rescued the non-invasive phenotype of the *amt1Δ.* This suggests that Amt1 may function to regulate cAMP during invasion (Mitsuzawa 2006).

1.5.8: Invasion and mating are both induced by low nitrogen

Invasive growth is induced on low carbon, high glucose media (Amoah-Buahin et al. 2005). Mating is induced on low-nitrogen media (Xue-Franzen et al. 2006). Both mating and invasion are responses to poor environmental conditions, however mating is a more extreme response. This is reflected by the levels of nitrogen that induce the two events. When nitrogen levels are low invasion is induced and the cells forage for a better supply by forming invasive structures. When nitrogen levels are extinguished mating is induced and the cells sporulate and effectively hibernate until favourable conditions return. The induction of mating results in repression of the cAMP signalling cascade and activation of the Byr2 mitogen activated protein kinase (MAPK) cascade (Mochizuki et al. 1992; Banuett 1998). Conversely, induction of invasion requires high glucose which activates the cAMP signalling cascade (Amoah-Buahin et al. 2005). This suggests that regulation of invasion must involve collaboration of other signalling events to elicit the response.

1.6: Invasive growth of other yeasts

Multicellular, invasive growth is exhibited by many ascomycetes (Stajich et al. 2009). This growth form has been widely studied in other organisms, however none have the extent of resources available as that of *S. pombe*. Despite this, the data from other organisms may help in understanding the process in *S. pombe.*

1.6.1: *S. cerevisiae*

S. cerevisiae is a budding yeast that is distantly related to *S. pombe* and is another popular model organism.

1.6.1i: *S.cerevisiae* common laboratory stock is non-invasive

S. cerevisiae forms pseudohyphal growth in the haploid and diploid genetic state. The stimulants vary for haploid and diploid cells (Gimeno et al. 1992; Roberts et al. 1994). Original research into *S. cerevisiae* pseudohyphal growth characterised it as a diploid specific growth form that did not occur in the majority of laboratory stocks. The inability of most laboratory stocks to undergo the pseudohyphal growth form was later traced to a nonsense mutation in FLO8 gene, which is a transcription factor required for invasive growth. A single base change from G to A in the FLO8 gene results in a premature STOP codon which manifests itself as a truncated version of the FLO8 protein which is subsequently unable to transduce the invasive signal (Liu et al. 1996).

1.6.1ii: Diploid *S. cerevisiae* form invasive and surface pseudohyphae

During nitrogen depleted conditions diploid cells, though not haploid, form pseudohyphal structures which spread across the agar and are capable of invasion (Gimeno et al. 1992). Conversely on nitrogen rich, low glucose media haploid cells, though not diploids, are able to invade but form limited filamentous structures on the agar surface (Roberts et al. 1994). The switch to pseudohyphal growth for diploid *S. cerevisiae* is associated with a number of cellular changes, most noticeably the cell morphology; pseudohyphal cells are significantly elongated and thinner in comparison to regular yeast cells and have a shorter G1 phase of the cell cycle (Gimeno et al. 1992). The formation of these pseudohyphal increases proportionally with cell density and a higher cell density produces stronger cell-surface adhesion. Therefore, the cell density of *S. cerevisiae* affects the pseudohyphal potential (White et al. 2011).

Cellular division during pseudohyphal growth is symmetric (Kron et al. 1994), with daughter bud growth reaching equal size to the mother cell before cell separation. This is in contrast to single cells where the daughter bud separates from the mother cell then enters a long G1 to attain critical growth size before passing START and entering a new cell cycle (Kron et al. 1994). Pseudohyphal growth requires a unipolar budding pattern of the leading filament tip, with decreased budding of the lateral daughter cells noted. Additionally the cells do not completely separate as they can be manipulated as units under the agar surface (Gimeno et al. 1992).

1.6.1iii: Haploid *S. cerevisiae* form invasive pseudohyphae

Haploid invasive growth varies slightly from diploid growth. Haploids invade nitrogen rich, glucose depleted media as quickly as three days under invasive conditions (Roberts et al. 1994). Fusel alcohols have also been shown to induce haploid invasion (Dickinson 1996). Fusel alcohols are metabolic by-products and are thought to induce growth in response to growth rate (Dickinson 2008). Haploids can form filaments on the agar surface, though these are not as elaborate and do not stretch as far as pseudohyphal growth of diploids (Roberts et al. 1994). Additionally, the morphology of these cells is not as elongated as seen for pseudohyphal growth. Haploid cells normally bud in an axial pattern. During invasive growth this switches to bipolar budding. This switch in bud pattern is not essential for invasive growth, though it is required for surface filamentous growth of haploid cells (Roberts et al. 1994).

1.6.1iv: Flo11 is a transcriptional target of invasion associated signalling

FLO11 is the master flocculin responsible for invasive growth of haploid and diploid *S. cerevisiae.* FLO11 is a cell surface glycol-protein that is responsible for cell-cell and cell-surface adhesion (Lo et al. 1996). The cAMP and the STE11 MAPK signalling cascade both result in up-regulation of FLO11 transcription (Gancedo 2001). The FLO11 promoter is 2.8Kbp with multiple repression and activation regions. SLF1 associates with this region under non-invasive conditions and contributes to transcriptional silencing of FLO11. This silencing is de-repressed upon cAMP mediated signalling which removes SLF1 and allows unwinding of the tightly packed chromatin surrounding FLO11 thus removing repression of the invasive response (Rupp et al. 1999).

1.6.1v: cAMP mediated signalling activates FLO11

RAS2, a small G protein, activates both MAPK and cAMP mediated FLO11 activation (Rupp et al. 1999). RAS2 activates GPA2 which in turn activates adenylate cyclase (Lorenz et al. 1997). This then increases intracellular cAMP and signals through protein kinas A (PKA) to activate FLO8 and subsequently FLO11 (Rupp et al. 1999).
S. cerevisiae PKA consists of a catalytic subunit, encoded by either *TPK1/2/3* and a regulatory subunit encoded by *BCY1* (Toda et al. 1987; Vinod et al. 2007). TPK2 is associated with pseudohyphal activation whereas TPK1 and TPK3 inhibit this growth

form. Activation of adenylate cyclase and subsequent increased intracellular cAMP causes activation to TPK2 via disassociation from BCY1. TPK2 then activates FLO8, which targets FLO11 and also inhibits SFL1, which results in repression of FLO11 inhibition. There is a negative feedback loop through cAMP mediated signalling which ensures the signal is not constitutively on. TPK2 also activates PDE1 and PDE2 which are phosphodiesterases responsible for the degradation of the cAMP signal (Vinod et al. 2007) (figure 1.8).

1.6.1vi: The STE11 MAPK mediated signalling cascade also activates FLO11

RAS2 also activates the MAPK phosphorylatory signalling cascade of STE20, STE11, STE7 (Mosch et al. 1996). This transduces the signal to the transcription factor STE12. RAS2 activates CDC24 which is the guananine nucleotide exchange factor (GEF) for CDC42 . GTP bound CDC42 then interacts with and activates STE20, which then sets of the phospho-relay of activation down the signalling cascade (Mosch et al. 1996). These are the same downstream signalling components responsible for the induction of mating specific response to pheromones a and α pheromone (Liu et al. 1993). During the mating response a and α factors bind to G protein-coupled receptors on opposite mating type cells (STE3 and STE2 respectively) which activates the alpha subunit (GPA1) and subsequent STE2O signalling cascade (Liu et al. 1993). The deletion of STE2/3, GPA1, STE4 (β subunit) or STE18 (γ subunit) does not affect the invasive growth response, however the deletion of the downstream signalling components from STE20 results in loss of invasive growth (Liu et al. 1993).

Therefore the invasion associated MAPK signalling cascade must utilise a different receptor for STE20 activation. Two separate signals activate a common signalling pathway but result in different effects, therefore differentiation between these two signalling cascades is required. This specificity is achieved by requirement of TEC1 association to STE12 for the invasive response (Madhani et al. 1998) (figure 1.8). Additionally, STE7 has variable targets in each response (Liu et al. 1993).

1.6.1vii: Differentiation between the mating and invasive response is achieved by alternate STE7 targets

The pheromone mating response results in FUS3 activation whereas the invasive response results in KSS1 activation (Roberts et al. 1994). KSS1 is phosphorylated by

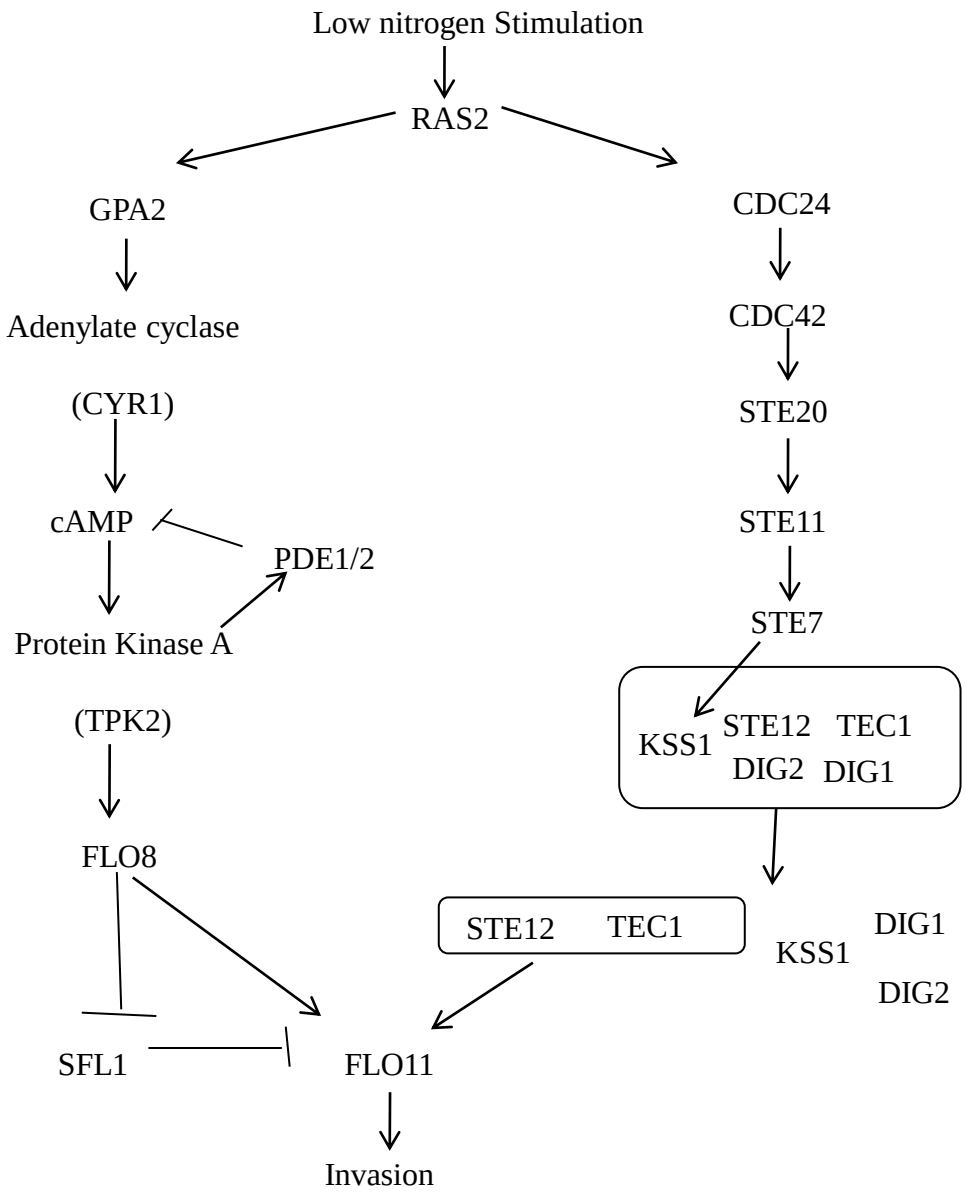


Figure 1.8 Signalling pathways that result in FLO11 activation in *S. cerevisiae.* RAS2 is activated by low nitrogen but the mechanism remains elusive. RAS2 then activates the cAMP signalling cascade as well as the MAPK pathway. STE7 phosphorylates KSS1 which causes phosphorylation of DIG1 and DIG2 and disassociation of KSS1, DIG1 and DIG2. STE12 and TEC1 are then free to activate FLO11.

STE7, prior to this KSS1 functions as an inhibitor of STE12 activity by recruiting DIG1 and DIG2 in a complex with TEC1 and STE12 (Gancedo 2001). DIG1 and DIG2 are inhibitors of the invasive response (Cook et al. 1996). Upon phosphorylatory activation of KSS1 by STE7, KSS1 phosphorylates DIG1 and DIG2 which cause their dissociation from this complex and allows the STE12/TEC1 complex to bind to filamentous response elements (FREs) within the promoters of target genes, one of which is FLO11 (Gancedo 2001) (figure 1.8). FREs have a STE12 consensus binding sequence (which is also found in the promoters of all mating pheromone activated genes) of TGAAACA with a TEC1 consensus binding sequence (CATTCPy) in close proximity. The presence of these two consensus binding sequences in close proximity facilitates STE12/TEC1 binding and subsequent transcriptional activation (Gancedo 2001) (figure 1.8).
Although FLO8 mediates the cAMP transduced signal to FLO11 and KSS1 mediates the STE20 MAPK signal there is crosstalk between these pathways, with the deletion of one pathway compensated for by the over-expression of the other pathway (Vinod et al. 2007).

1.6.1viii: Large scale approach to identifying *S. cerevisiae* strains that exhibit an aberrant invasive phenotype

Two large scale approaches have been used to study filamentous growth in *S. cerevisiae.* Firstly analysis of the kinome revealed alteration of protein localisation. Six kinases localize to the nucleus during filamentous growth, in contrast to their cytoplasmic location during vegetative growth. These six kinases are involved in a network to allow filamentous growth that is not properly understood (Bharucha et al. 2008).
The second large scale approach involved gene disruption and over-expression analysis. This screen identified 487 genes that may be involved in filamentous growth and implicated the mitochondrial retrograde signalling pathway as a negative regulator of the process (Jin et al. 2008). The large data set is an example of why large scale screens are a good approach to decipher the cellular processes responsible for a phenotype. However, this *S. cerevisiae* screen only focused on the invasive efficiency and colony morphology of the gene disruptants. It did not note the morphology of the invasive filaments of the mutants, therefore missing any genes that are involved in regulation of filament morphology.

1.6.2: *Schizosaccharomyces japonicus*

Schizosaccharomyces japonicus (S. japonicus) is a member of the *Schizosaccharomyces* genus alongside *S. pombe. S. japonicus* is capable of hyphal and pseudohyphal growth forms (Sipiczki et al. 1998) which was initially thought to be limited to growth on solid media but more recently liquid filamentation has been described (Furuya et al. 2010). Pseudohyphae form on the surface of the agar and once they penetrate the agar the cells enter true hyphal growth phase (Enczi et al. 2007). *S. japonicus* can switch to hyphal growth on most media over a broad range of temperatures (19-35ºC), with hyphal structures first visible after six days incubation (Sipiczki et al. 1998).

1.6.2i: *S. japonicus* switch to hyphal growth as an escape response

The invasive switch is thought to be driven by a nutrient gradient and be predominantly due to nitrogen deprivation (Sipiczki et al. 1998). Invasive filaments only grow towards a fresh media source; filaments do not grow towards other filaments. The sensing mechanism behind this process is still elusive. As these cells only grow towards fresh nutrients it suggests they moving away from poor nutrient conditions in search of better ones (Sipiczki et al. 1998).

1.6.2ii: Large vacuoles function in invasive growth of *S. japonicus*

The hyphal cells grow via monopolar extension as shown by staining of actin and growth sites (Sipiczki et al. 1998). *S. japonicus* undergoes a gradual conversion from bipolar to monopolar growth which correlates with mass vacuolation at the non-growing end (Enczi et al. 2007). This vacuolation serves two purposes. Firstly it acts to increase the cell volume to meet with the demands of the larger, extended cell thus concentrating the cytoplasmic content at the growing tip. The large vacuoles are also thought to repress growth from the rear end of the cell. Hyphal cells divide by medial fission every 2.3 hours. When this occurs the large vacuoles are not shared equally between the two daughter cells. However, the vacuolation quickly reappears at the new end of the leading hyphal cell post cell division (Enczi et al. 2007). Prior to cell division small vacuoles are shuttled along microtubules from the rear of the leading hyphae to the centre; the area which will be the rear of the leading hyphae post cell division. These small vacuoles are the precursors for the large vacuoles that appear quickly after cell separation (Sipiczki et al. 1998).

1.6.2iii: Microscopy of *S. japonicus* hyphae

Microtubule staining in *S. japonicus* reveals microtubules that stretch from the leading hyphae tip down to the large vacuoles at the non-growing end (Sipiczki et al. 1998). Microtubules are involved in determining growth polarity by serving as tracks for the delivery of growth components. Therefore, the large vacuoles at the non-growing hyphae end prevent delivery of growth components (Sipiczki et al. 1998). The invasive hyphae of *S. japonicus* form septa but these do not undergo full cell separation. Electron micrographs of these septa revealed a lack of pores in these septa therefore no cytoplasmic flow occurs (Sipiczki et al. 1998). DAPI staining revealed the filaments of *S. japonicus* hyphae are mono-nucleate (Sipiczki et al. 1998).

1.6.2iv: Signalling pathways involved in hyphal formation of *S. japonicus*

Hyphal formation of *S. japonicus* involves cAMP mediated signalling.
However, in contrast to *S. cerevisiae* and *S. pombe* where increased cAMP results in invasive growth, a decrease in cAMP appears to be crucial for invasive growth in *S. japonicus* (Sipiczki et al. 1998). Sipiczki *et al*. (1998), supplemented growth media with a cAMP analogue, glucose (which induces cAMP production) and caffeine (which inhibits phosphodiesterases responsible for cAMP degradation therefore causing a rise in cAMP levels). All three inhibited hyphal formation suggesting a decrease in intracellular cAMP is required for the switch from yeast to hyphal growth in *S. japonicus* (Sipiczki et al. 1998).
More recently Furuya *et al.* (2010), have suggested the DNA damage checkpoint repair pathway is involved in the yeast to hyphal switch. Treatment of cells with a DNA damage inducing agent camptothecin (CPT), induces hyphal formation. This response is not solely brought about by CPT and can also be seen by addition of MMS, bleomycin and gamma rays; these all induce DNA damage (Furuya et al. 2010). Additionally *rad51Δ,* which has a high rate of spontaneous DNA damage as it is required for recombination dependent DNA repair (Krogh et al. 2004), is hyper-invasive. They trace this response to Chk1, a kinase downstream of the DNA damage checkpoint. *Chk1*Δ cells are unable to respond to CPT induced hyphal growth, however *chk1*Δ cells are capable of non-CPT induced hyphal growth implying it is not essential for hyphal formation (Furuya et al. 2010). This type of DNA damage dependent filamentous stimulation is not seen in *S. pombe* or *S. cerevisiae.*

1.6.2v: Deletion strains that exhibit a non-invasive phenotype

Random mutagenesis identified seven genes of *S. japonicus* that exhibit an aberrant invasive phenotype. These were named the *myc* (mycelia) genes (Enczi et al. 2007). The deletion of these genes results in a range of phenotypes including short bipolar growing cells, round isotropically growing cells as well as chains of cells (Sipiczki et al. 1993; Enczi et al. 2007). These multiple phenotypes suggest multiple pathways regulate hyphal formation. This random mutagenesis approach presumable only identifies a small number of genes involved in the invasive response. A larger number of genes would be identified by a functional genomics screen, but as yet this is not applicable to *S. japonicus* as the resource is not available.

1.6.3: *Candida albicans*

Candida albicans (*C. albicans*) is an opportunistic pathogen (Datta et al. 1989).

1.6.3i: Multiple growth forms of *C. albicans*

It is a trimorphic ascomycete capable of yeast, pseudohyphal and true hyphal growth (Whiteway et al. 2007). Pseudohyphae are long branched strings of connected ellipsoidal cells that exhibit a unipolar budding pattern whereas true hyphae are cylindrical filaments composed of elongated cells with septa that undergo consistent polarised growth. *C. albicans* can form filaments in serum as well as embedded in media. Environmental factors such as temperature, O_2 availability, pH and nutrient availability are all involved in the induction of a switch in growth form (Whiteway et al. 2007). The hyphal growth phase of *C. albicans* is not exclusive. The sub-apical region of the cell is a growth site of lateral yeast cells (Shen et al. 2008). The switch in growth pattern from yeast to hyphal is crucial in virulence as shown by deletion strains that exhibit an inhibition of filamentation which coincides with avirulent phenotypes (Hayek et al. 2009). Additionally the switch from hyphal back to yeast form is as important, as constitutively filamentous strains also display avirulence (Shen et al. 2008).

1.6.3ii: Invasive growth of *C. albicans* involves enzymatic secretion and the actin cytoskeleton

The apical tip of *C. albicans* contains a Spitzenkorpe. This is an aggregation of vesicle structures that excretes enzymes which facilitate the invasive response (Whiteway et al. 2007). The importance of the actin cytoskeleton during hyphal formation is emphasised

by treatment with latrunculin A which depolymerises the actin cytoskeleton and abolishes hyphal growth (Hazan et al. 2002).

1.6.3iii: Signalling pathways involved in invasive growth of *C. albicans*

There have been no large scale reverse or forward genetics approaches to identify genes involved in the invasive response of *C. albicans.* Genes have been identified by comparative analysis of invasive genes of *S. cerevisiae.* As in *S. cerevisiae* increase of intracellular cAMP results in invasive growth. This is achieved via signalling through GPR1, a putative G protein-coupled receptor and GPA2, its associated α subunit (Miwa et al. 2004). These then transduce the signal through TPK1 and TPK2 which are protein kinase A catalytic components (Bockmuhl et al. 2001). This signalling is thought to feed down through CDC42 and other signalling components to HWP2, which is the *C. albicans* homologue of *S. cerevisiae* FLO11, a cell surface glycoprotein that is essential for invasive growth (Hayek et al. 2009). Glycoproteins are thought to serve a major role in invasive growth with varying roles associated with lipases, proteases and adhesin function. No such glycoprotein has been identified in *S. pombe* . It seems likely that such a glycoprotein is involved in *S. pombe* invasion and hopefully further investigation may identify one.

The pheromone induced mating MAP kinase signalling cascade (CPH1, HST7, STE20) is also essential for hyphal growth in *C. albicans* (Lengeler et al. 2000).

1.6.3iv: Different *C. albicans* growth forms required different signalling cascades

There are multiple transcription factors that are important during variable conditions that induce the *C. albicans* filamentous response. The transcriptional activators for the serum response, Efg1 and Flo8, are targets for cAMP mediated signalling (Whiteway et al. 2007). These are different from the regulatory proteins involved in embedded hyphal formation which requires that Efg1 cAMP mediated signalling is turned off. pH induced hyphal formation is regulated by Rim101, a transcription factor that is activated by proteolytic cleavage by Rim13 protease (Braun et al. 1997). Upstream factors of this proteolytic cleavage, Rim20, Rim8 and a potential membrane sensor Dfg16 have been identified though the pathway details remain unclear (Braun et al. 1997).

1.7: Signalling processes of *S. pombe*

There are multiple well-documented signalling cascades in *S. pombe*¸ including three mitogen activated protein kinase (MAPK) cascades, however only those relevant to invasive growth shall be discussed.

1.7.1:Glucose induced cAMP signalling

Cells respond to environmental cues to ensure they are utilizing the best of their surrounding environment. As such when glucose levels are high cells do not waste valuable energy on the production of glucose from carbon substrates therefore gluconeogenesis is transcriptionally repressed. High glucose additionally informs the cell that the environment is rich in carbon source so sporulation is not necessary and therefore repressed (Hoffman 2005).

1.7.1i: *fb1-ura-lacZ* fusions facilitated investigation of the glucose activated cAMP signalling cascade

Study of this glucose regulated pathway was facilitated by the fusion of a downstream transcriptional target, *fbp1*, to reporter genes ura4 and lacZ. The *fbp1-ura4* fusion allowed identification of mutants in this pathway via selection of prototrophic uracil colonies, and the *fbp1-lacZ* fusion facilitated quantitative analysis of the genes (Hoffman et al. 1990). *fbp1* is a gluconeogenic gene that is transcriptionally repressed when glucose levels are high (Vassarotti et al. 1985). To identify genes involved in this repression Hoffman *et al*. (1990), looked for spontaneous mutations that resulted in uracil prototrophy in strains transformed with the *fbp1-ura4* fusions under glucose repressing conditions. In these high glucose conditions *fbp1-ura4* transcriptional would normally be repressed. However if a spontaneous mutation created a problem in the repressive pathway then constitutive expression of the *fbp1-ura4* fusion would be seen and colonies would be selectable as uracil prototrophs. This methodology was used to isolate 8 glucose insensitive transcription (*git*) genes (Hoffman et al. 1990).

1.7.1ii: *git* genes function in cAMP mediated signalling

Further research indicated that glucose mediated repression of *fbp1* is dependent on cAMP mediated signalling (Hoffman et al. 1991). Glucose binds to the G protein coupled receptor Git3, which associates with Gα and GβGγ subunits, encoded by *git2*, *git5* and *git11* respectively (Welton et al. 2000). These are located on the internal face of the plasma membrane. Glucose binding to Git3 results in a confirmation change

which causes an exchange of GDP for GTP by Gα. This subsequently causes the GβGγ subunits to dissociate from the Gα subunit. This results in adenylate cyclase (Git2/Cyr1) activation and an increase in intracellular cAMP (Hoffman 2005).

The G protein coupled receptor (Git3), its associated proteins (Git5, Git8/Gpa2 and Git11) and Git2/Cyr1 account for four of Hoffman *et al*.'s (1990) originally described *git* mutants. Git11 was later identified as the γ subunit through a two-hybrid screen for a binding partner for Git5 (Landry et al. 2001). *Git6/pka1* encodes the cAMP activated protein kinase A (PKA) catalytic subunit, which is responsible for downstream activation of the signalling cascade (Byrne et al. 1993).

1.7.1iii: Three *git* genes have unknown functions in cAMP mediated signalling

The remaining three *git* mutants do not have such clear functions during the glucose repressive response. These mutants cannot be rescued by over-expression of Gpa2, thereby implying they work downstream of the Gα subunit or in a alternative pathway that results in adenylate cyclase activation. Git1 is a multi-domain protein that has no obvious homologue in other eukaryotes. Co-immunoprecipitation experiments have shown it physically binds to adenylate cyclase, though *git1* deletion strain analysis reveal it is not required for localisation or stability of adenylate cyclase (Kao et al. 2006). During analysis of adenylate cyclase localisation they noted it was not often seen at the membrane. The C2 domain of Git1 has been shown to promote association with phospholipids in a calcium dependent manner and the weakly conserved Munc homology domains (MDH1, MDH2) are thought to function in priming vesicles for cellular fusion (Kao et al. 2006). Site-directed mutagenesis has shown that both these domains are required for the Git1 function in glucose repression, and Kao *et al*. (2006) suggest this may be related to the MDH domains which may facilitate vesicle associated transient migration of adenylate cyclase to the membrane for interactions and activation with Gα subunit.

git10 is identical to *hsp90*, the heat shock chaperone protein (Hoffman 2008). Hoffman *et al*. (2008), identified a separation of function mutation within Git10 that results in loss of glucose repressive but not heat-shock chaperone function. The mutation is in the central domain that is thought to be for client protein binding (Hoffman 2008). Git10 may function in association with Git7 during the glucose repressed response. Git7 is an

Sgt1 family member, its *S. cerevisiae* homolog is also involved in cAMP signalling though their roles remain unclear (Hoffman 2005).

1.7.2: Calcineurin is a protein phosphatase

Calcineurin is a Ca^{2+}/calmodulin dependent type 2B serine/threonine protein phosphatase that is highly conserved between yeast and mammals (Yoshida et al. 1994). Calcineurin is dispensable for vegetative growth, but crucial in response to environmental stress and is known to function in a wide range of cellular processes including ion homeostasis and morphogenesis (Yoshida et al. 1994; Sugiura et al. 1998). Calcineurin is functionally active as a hetero-dimer consisting of a 61kDa calmodulin binding catalytic subunit A and 19kDa Ca^{2+} binding regulatory subunit B (Yoshida et al. 1994). Calmodulin binding to the catalytic subunit A is required for full activation but can only occur in the presence of Ca^{2+} bound to the regulatory subunit B. *S. pombe* has a single gene encoding the catalytic subunit, *ppb1* and the regulatory subunit, *Spcc830.06* (Yoshida et al. 1994; Sio et al. 2005). Deletion of the catalytic and regulatory subunits of calcineurin result in the same hypersensitivity to Cl^-. Additionally over-expression of the catalytic subunit does not rescue phenotypic aberration of the regulatory subunit deletion strain, implying they are both crucial for calcineurin function(Sio et al. 2005). Sio *et al.* 2005 suggest the activation of the catalytic subunit may be facilitated by a conformational change in the regulatory subunit brought about by the binding of Ca^{2+}.

1.7.2i: Calcineurin activation

The signalling process that leads to increased Ca^{2+} and therefore calcineurin activation is not clearly understood but is thought to occur via two independent Ca^{2+} mediated pathways as shown by differential real time calcineurin activation profiles and epistatic phenotypic results (Deng et al. 2006). Addition of $CaCl_2$ to the medium results in a high Ca^{2+} extracellular environment, which in turn activates calcineurin by an increase in intracellular Ca^{2+}. This increase in intracellular Ca^{2+} is thought to be facilitated by transcriptional up-regulation of vacuole and Golgi Ca^{2+}/ATPase pumps, *pmr1* and *pmc1* respectively (Deng et al. 2006).*Vcx1* a vacuole H^+/Ca^{2+} exchanger which pumps Ca^{2+} into the cytoplasm (Miseta et al. 1999), is thought to maintain Ca^{2+} homeostasis and be subject to transcriptional up-regulation. Calcineurin is also activated by treatment with drugs that cause cellular damage such as DTT and tunicamycin (ER stress), Micafungin

(cell wall defects) as well as addition of NaCl and KCl (Deng et al. 2006). The activation profiles, as determined by a luciferase reporter fused to a calcineurin dependent response element (CDRE) which were integrated into the *S. pombe* genome, vary between these different activating agents (Deng et al. 2006). This suggests the mode of activation also varies. *Yam8* and *cch1* encode putative components of a Ca^{2+} channel and deletion of these genes results in abolished calcineurin activation caused by cell wall defects or treatment with NaCl, though not of treatment with $CaCl_2$ (Deng et al. 2006). This strengthens the proposal that calcineurin is activated by two separate pathways, one that involves the Pmc1, Pmr1 pumps and one that utilizes a potential Yam8, Cch1 Ca^{2+} channel. Additionally deletion of components of the Pmk1 cell integrity response pathway abolish Yam8, Cch1 mediated calcineurin activation and over expression of Pmk1 induces a high level of calcineurin activity thus strongly indicating the Pmk1 MAPK cell integrity pathway activates Ca^{2+} signalling in these responses (Deng et al. 2006). This is in contrast to the proposed role of Pmk1 MAPK cascade in Cl^- homeostasis where it is proposed to function antagonistically (Sugiura et al. 1998).

1.7.2ii: Prz1 is a calcineurin target protein

Once activated, calcineurin de-phosphorylates downstream protein targets on serine or threonine residues. One well described target is the transcription factor Pzr1, a zinc finger transcription factor identified based on homology to *S. cerevisiae* CRZ1 (Hirayama et al. 2003). Upon de-phosphorylation this translocates to the nucleus and activates transcription of proteins with calcineurin dependent response element (CDREs) within their promoters (AGCCTC). The transcriptional regulatory profile of calcineurin is thought to encompass a broad range of targets including genes involved in ion transport and morphology (Hirayama et al. 2003).

In *S. cerevisiae* CRZ1 appears to be the main pathway of calcineurin mediated control (Stathopoulos et al. 1997), however this is not the case in *S. pombe*. There are a number of calcineurin associated deletion phenotypes, such as Cl^- homeostasis and morphological aberrations, that are not mimicked by the deletion of Pzr1 (Hirayama et al. 2003). This suggests that there are other functional signal transduction routes employed by calcineurin. Prz1 does regulate Ca^{2+} homeostatic response as Prz1 has been shown to regulate the mRNA levels of Pmc1, the Ca^{2+} pump (Deng et al. 2006).

1.7.2iii: Calcineurin functions in DNA damage induced NETO delay

More recently Kume *et al*. (2011) reported a crucial role of calcineurin in DNA damage mediated new end take off (NETO) delay which ensures the completion of DNA repair prior to the progression of the cell cycle (Kume et al. 2011). Although this effect was specifically seen under DNA damage conditions it identifies a direct role of calcineurin as a signalling molecule capable of controlling cell morphogenesis and polarised growth. Kume *et al*. (2011) described Tip1, a member of the +TIP complex responsible for determination of polarised growth site, as a direct target for calcineurin phosphatase activity and the dependence of this activity for the delay in NETO as a cellular response to DNA damage. This calcineurin function was independent of Pzr1 mediated response and represents a separate path of calcineurin signalling (Kume et al. 2011).
Kume *et al*. (2011) used FK506 to analyse the relationship between the *pol1Δ* (DNA damaged strain) induced NETO delay and calcineurin. FK506 is a calcineurin inhibitor (Ho et al. 1996), therefore treatment of wild type cells with FK506 mimic calcineurin deletion. They found that upon FK506 treatment the delay is attenuated, thereby implying calcineurin is a mediator of the signal. Co-immunoprecipitation experiments showed a physical association between Ppb1 and the microtubule motor Tea2, as well as the check point kinase Cds1 (Kume et al. 2011). Additionally, epistatic analysis facilitated by over-expression experiments allowed Kume *et al.* (2011) to conclude that Cds1 activates calcineurin which subsequently de-phosphorylates Tip1 and this is required for the delay of NETO after DNA damaged. This idea is supported by localisation of Ppb1-GFP to the interphase microtubules tips within DNA-damaged cells (Kume et al. 2011).

1.8: Functional genomics as an experimental method

The complete *S. pombe* genome was published in 2002 (Wood et al. 2002). This led to a new era of research which involved large scale genome analysis. Functional genomics approaches involve large scale investigations into the relationship between gene function and phenotype. These can highlight novel genes involved in processes which would otherwise remain undiscovered. These large scale analyses provide data sets which enable comparison to equivalent data sets from other organisms. This can aid phylogenetic analysis as well as identification of important genes within a process through conservation of phenotypes across species. The use of this large scale approach

has previously identified genes involved in invasion of *S. pombe* (Dodgson et al. 2009). These genes were identified via a screen of the Bioneer *S. pombe* deletion library.

1.8.1 The Bioneer *S. pombe* deletion library

The *S. pombe* deletion library was created by the Bioneer corporation (http://pombe.bioneer.co.kr/) and collaborators. Bioneer have made three version of the library, each with more strains than the last. The libraries consist of thousands of strains each with a single gene deleted.

1.8.1i: Deletion of the target gene

This gene deletion is achieved by replacement of the target ORF with Kanamycin resistance module. The replacement is facilitated by homologous recombination of the target with a DNA fragment that is transformed into the host strain. The DNA fragment contains a Kanamycin resistance module (KanMX4) which comprises the Kanamycin resistance ORF between a TEF promoter and terminator. The KanMX4 module is flanked by a unique upstream and downstream 20mer tag sequences which are themselves flanked by an upstream and downstream universal sequence. Finally flanking the entire module are 80bp sequences that are homologous to roughly 80bp upstream and downstream of the target ORF start and stop codon respectively. These allow homologous recombination upon host transformation and replacement of target ORF. The integration site of the DNA fragment that replaces the target ORF varies slightly from strain to strain (http://pombe.bioneer.co.kr/).

1.8.1ii: Conversion of the deletion libraries to haploidy

These DNAs were transformed into diploid strains that were auxotrophic for adenine, leucine and uracil. Their genotypes were h+/h+, ade6-M210/ade6-M216, ura4-D18/ura4D18, leu1-32/leu1-32, ORFΔ::KanMX4. The host strain used for the production of the deletion library was a non-sporulating diploid therefore to create a haploid version of the library the meiotic competence of these strains had to be restored. This was done by transformation with the pON177 plasmid (Styrkarsdottir et al. 1993). This results in sporulation and allows selection of haploid deletion strains. The pON177 plasmid is then exclude from the strains resulting in a haploid deletion library which is auxotrophic for adenine, leucine and uracil (http://pombe.bioneer.co.kr/).

1.9: A screen of the *S. pombe* deletion library for aberrant invasive phenotypes

Study of the invasive growth form is not only important for the investigations of pathogenicity in harmful organisms but it is also relevant to polarised growth. The aim of this thesis is to learn more about the changes that occur in the switch from unicellular to invasive growth in *S. pombe*. To do this, a screen of the Bioneer deletion was performed. The screening of these deletion libraries allows quick identification of genes involved with the invasive process via exhibition of aberrant phenotypes. This large scale approach may result in identification of novel genes that would not have been considered for forward genetics approaches. No large scale analysis has been performed on the morphology of invasive structures in other organisms therefore this could highlight novel phenotypes and mechanisms. Additionally, this screen was performed on low-nitrogen media to identify genes that may be involved in nitrogen sensing.
The roles of signalling pathways within the invasive process was investigate by supplementing growth media with exogenous compound or inhibitor as this has been previously shown to stimulate invasion (Amoah-Buahin et al. 2005; Prevorovsky et al. 2009). Finally, the role of spindle pole bodies and the septation initiation network was investigate during filament formation.

Chapter 2: Materials and Methods

2.1: Materials

2.1.1: Media for growth of *S. pombe*

2.1.1i: Yeast extract plus supplements (YES) (+G418) media

Per litre: 5g yeast extract, 30g glucose, 100mg adenine, 100mg leucine, 100mg uracil, 100mg lysine, 100mg histidine, 20g agar (for solid media) + 500µL of 100mg/ml G418 stock solution for 50mg/l YES + G418 (Melford; http://www.melford.co.uk/) media.

2.1.1ii: Edinburgh Minimal Media (EMM)

Per litre: 3g potassium hydrogen phthalate, 2.2g disodium hydrogen phosphate, 5g ammonium chloride, 20g Glucose, 1ml 1000x PM Vitamins, 0.1ml 10,000 x PM minerals, 20ml 50 x PM Salts, 20g agar (for solid media), 0.75g supplements (uracil, adenine, histidine, leucine and lysine) were added when required from 1.5mg/ml stock solutions

2.1.1iia: EMM + 5FOA + uracil

As above but including 5FOA to 0.25% (from 100mg/ml stock) and 0.075g/l uracil (from 1.5mg/ml stock). The plates were stored at 4ºC in the dark.

2.1.1iii: Low Nitrogen Base (LNB) media

Per litre: 67mg yeast nitrogen base, 20ml 50x PM salts, 50ml 20% glucose, 1ml 1000x PM vitamins, 10g agar (for solid media).

2.1.1iiia: Preparation of LNB media

LNB plates must be left to set with their lids on. Following this they must be left to dry with their lids off in the 37ºC incubator of at least 6 hours.

2.1.1iiib: 0.5µg/ml FK506 LNB media

LNB was made as above but 0.5µg/ml FK506 was added.

2.1.1iiic: 4mM $FeCl_2$ LNB media

LNB was prepared as described above with the addition of $FeCl_2$ to the concentration of 4mM.

2.1.1iiid: Pyridoxal-5-phosphate LNB media

LNB was prepared as described above with the addition of pyridoxal-5-phosphate to the concentration of 1μM, 20μM or 100μM.

2.1.1iiie: Pyridoxal LNB media

LNB was prepared as described above with the addition of pyridoxal to the concentration of 100μM.

2.1.1iv: SPAS mating media

Per litre: 10g glucose, 1g KH_2PO_4, 1ml 1000 x PM vitamins, 45mg supplements (uracil, adenine, histidine, leucine and lysine) were added when required from 1.5mg/ml stock solutions, 20g agar.

2.1.2:Media for growth of *E. coli*

LB media, per litre: 10g Tryptone, 5g yeast extract, 10g NaCl

2.1.3: Buffers and solutions

All solutions and buffers were filter sterilised prior to use.

Adenine, Leucine, Uracil, Histidine and Lysine 20x stock solutions: Per 500ml: 0.75g.

PPN 20x: Per litre: 60g phthallic acid, 36g Na_2HPO_4, 100g NH_4Cl.

PM Salts (x50): Per litre: 53.5g $MgCl_2.6H_20$, 0.75g $CaCl_2.2H_20$, 50g KCl, 2g Na_2SO_4.

PM minerals (x1000): Per 500ml: 2.5g boric acid, 2g$MnSO_4$, 2g $ZnSO_4$, 1g $FeCL_2.6H_20$, 0.8g H_2MoO_4, 0.5g KI, 0.2g $CuSo_4.5H_20$, 5g citric acid.

PM vitamins (x1000): Per litre: 1g pantothenic acid, 10g nicotinic acid, 10g inositol, 10mg biotin.

LiAc/EDTA (100ml**):** 1.02g LiAc, 100ml distilled H_20, 200μl of 0.5M EDTA.

PEG solution (100ml): 1.02g LiAc was added to 50ml distilled H_20, 200μl of 0.5M EDTA was added and adjusted to pH 4.9 with acetic acid. 40g PEG 3350 was added. The solution was made up to 100ml with distilled water and heat was applied to dissolve the PEG.

0.5 M Potassium phosphate buffer: 61ml 1M K_2HPO_4, 39ml 1M KH_2PO_4

1M K_2HPO_4: 10.62g K_2HPO_4 in 61ml distilled water.

1M KH_2PO_4: 5.31g KH_2PO_4 in 39ml distilled water.

PEM buffer, 100mM PIPES, 1mM EGTA, 1mM $MgSO_4$: 1.73g PIPES was dissolved in 45ml H_2O and adjusted to pH to 6.9. 19mg EGTA and 12.3mg $MgSO_4$ were added.

SP1, pH 5.6: 1.2M sorbitol, 50mM sodium citrate, 50mM sodium phosphate, 40mM EDTA.

5M Potassium acetate: 4.9g KAc in 10ml distilled water.

5FOA (100x) : 100mg/ml 5FOA in DMSO.

Calcofluor: 35mg Calcofluor in 7ml distilled H20. A couple of drops NaOH were added to dissolve the Calcofluor. This was adjusted to 10ml with water and stored at -20ºC. The tube was wrapped in tin foil as Calcofluor is sensitive to light.

0.1M 8-Br-cAMP: 5mg 8-Br-cAMP in 122µl distilled water.

100mg/ml G418: 1g G418 in 10ml water.

Zymolyase (Seikagaku corporation): 20mg/ml in water

1 x TE, pH 8: 10mM TRIS buffer, adjusted to pH8 with HCL, 1mM EDTA

10x TBE Per Litre: 108g Tris base, 55g Boric acid, 9.3g EDTA

100bp DNA ladder solution: 4µl water, 1µl 6x loading dye, Blue (NEB), 1µl 100bp DNA ladder (NEB).

2.1.3i: Source of chemicals

All chemicals were purchased from Fisher scientific (http://www.fisher.co.uk/) or Sigma Aldrich (http://www.sigmaaldrich.com/united-kingdom.html) unless otherwise stated. Yeast nitrogen base was purchased from Formedium (http://www.formedium.com/uk). Agar was purchased from B.T.P Drewitt, London, UK.

2.2: Common methods

2.2.1:Mating single colonies

Each parent strain was grown for 3 days at 30ºC. 10µl distilled water was pipetted into an eppendorf tube and an inoculation loop was used to scrape some fresh cells of each parent into the 10µl water. These cells were thoroughly mixed before being transferred to an SPAS mating plate. These plates were incubated at 27ºC for 3 days. After 3 days the mating mixtures were inspected microscopically for asci. If plenty of asci were present the mating mixtures were prepared for their subsequent purpose; immediate

transfer to selection plates, enzyme digestion for random spore analysis or transfer to plates for tetrad dissection.

2.2.2: *S. pombe* DNA extraction

10ml YES broth was inoculated with a 1µl inoculation loop-full of fresh cells. This was incubated overnight at 30ºC. 1ml of overnight innoculum was transferred into a 2ml Eppendorf tube. This was centrifuged for 5 minutes at 6500 rpm. The supernatant was discarded. A further 1ml was transferred to the same tube. This was centrifuged for 5 minutes at 6500 rpm. The cells were re-suspended in 250µl of SP1 solution. Zymolyase was added to the final concentration of 0.4mg/ml. Cells were mixed by pippetting and incubated at 37ºC for 30 minutes. 10µL of digestion mixture was transferred to a microscope slide. 1µl of 10% SDS was added and the cells were inspected with a light microscope. The spheroplasted cells appeared darker than the live cells. If roughly half the cells had been spheroplasted the cells were pelleted for 15 seconds at 6500 rpm. The supernatant was discarded and the pellet was re-suspended in 0.5ml 1x TE. This was vortexed briefly. 0.5ml 10% SDS was added and tube was gently rotated. This was left at room temperature for 5 minutes. 165µl 5M potassium acetate was added and left on ice for 30 minutes. The tubes were occasionally rotated. After 30 minutes the tubes were centrifuged at 13000 rpm for 10 minutes. The supernatant was transferred to a sterile new tube and 750µL cold isopropanol was added. The tubes were left on dry ice for 5 minutes. The tubes were then centrifuged at 13000 rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 200µL sterile water. RNase was added to 10µg/ml from the 10mg/ml stock solution. This DNA could be used as a template for PCR or could be stored at – 20ºC .

2.2.3: Confirmation of strains by PCR

PCR was used to confirm the identity of a selection of strains. Forward primers were designed by choosing a 20bp region 200bp upstream from the start codon of the specific gene. Primer design was facilitated by Artemis, a software programme that displays the annotated *S. pombe* genome (Rutherford et al. 2000). A reverse primer was used that is homologous to a region 718 downstream of the Kanamycin resistance start site (designed by James Dodgson). The PCR reaction mixture was made to a final volume of 20µL.

Component	Volume (µL)
2mM dNTPs	2
25mM $MgSO_4$	2
10 x Buffer for KOD Hot Start DNA polymerase	2
1 U/µl KOD Hot Start DNA polymerase	0.4
10µM up-primer	0.2
10µM down- primer	0.2
Template DNA	1
Water	12.2

The reagents were purchased from Invitrogen (http://www.invitrogen.com). The reagents were thoroughly mixed on ice. A Biometra thermocycler (http://www.biometra.de/) was used. The PCR cycle used was;

Step1 - 95ºC for 2 minutes

Step2 – 95ºC for 20 seconds
Step3 – 60ºC for 10 seconds
Step4 – 70ºC for 15 seconds
} repeated 30 times

2.2.3i: Primers

Primer	Sequence (5' – 3')
SPAC3H5.11	GTACGCATCCACCACTTTCC
SPBC18H10.19	GTCACGAAGAGTACTTTATC
Rad25	GGCATTTGTTTTTGGAAGCC
SPBC3B8.08	CCAAAACTTTCAGTGCAGCG
SPBC1539.06	GAGTATTGCAAAGTCTGTGG
SPCC1322.07c	GTGTACAATGAGATCGACGC
Ace2	CAGCGCACCAAACATTGCTG
Sty1	GGATACGAGGTCCGATACC
Rad24	GAGAACAGTCCAGCAGCCAG
Alg10	GTG TGC ATAATG TTG AAT GG
Gpd2	CAC TCA CTT ACA TTA GTA GG
SPCC18.10	AAT ATC AGG TTA ATC AAA GC

Tip1	CTT CTT TTA ATT CTT CTC CC
Tea2	CAT AGT TTA CTG GTT ATT CG
Mbo1	AAA ATA TCT GCT TCT AAC AC
Kes1	GCC GGC GAC TAT GCA GCT GC
Asl1	CGG CTT GRC GTT CAC CAA CC
Fkh2	CCG GTA TCT TGA TTG TTT AC
KanR	TCA GCC AGT TTA GTC TGA CC

2.2.4: Gel electrophoresis

0.3g agarose was dissolved (with heat) in 30ml 1 x TBE buffer. 1µL Gel Red (Biotium, http://www.biotium.com/) was added to the molten solution. This was poured into a cast and allowed to set for 10 minutes.

The gel was transferred into the electrophoresis apparatus and submerged in 1 x TE buffer.

Sample preparation: PCR product was mixed with loading dye and 1 x TE to a final volume of 10µL.

Component	Volume (µL)
PCR product	6
10x TE buffer	2
Gel loading dye, Blue (6x) (NEB)	2

The samples were loaded. The gel was run at 120V for 60 minutes or until the Bromophenol Blue of the samples was close to the end of the gel. A 100bp DNA ladder (NEB) was used.

The gel was then observed under the Eagle Eye UV transilluminator.

2.2.5: QIagen miniprep.

QIAprep spin miniprep kit (http://www.qiagen.com) was used to extract plasmid DNA from Dh5α competent cells (Invitrogen).

2.2.6: Restriction enzyme digestion

The digest was performed in a 50µL final volume. The plasmid DNA extracted from QIagen minipreps was digested with Not1.

Component	Volume (µL)
10x NE Buffer 3 (NEB)	5
100µg/ml BSA	0.5
Plasmid DNA (from plasmid extraction)	43.5
10,000 u/ml Not1 restriction enzyme	1

These components were mixed in an Eppendorf and incubated for 1 hour at 37ºC.

2.2.7: Transformation

Cells were grown overnight in 10ml YES. 2ml of overnight cell suspension was centrifuged for 1 minute at 6500rpm. The supernatant was discarded. The pellet was re-suspended in sterile water at roughly 10^8 cells/ml. 1ml of cell suspension was transferred to a sterile 1.5ml Eppendorf tube. 0.2ml LiAc/EDTA was added. This was vortexed and centrifuged for 1 minute at 6500rpm. The supernatant was discarded. The pellet was re-suspended in 50µl LiAc/EDTA. 500ng DNA that had been extracted via miniprep was added to the suspension. 300µl PEG was added. This was incubated with agitation at 30ºC for 30 minutes, followed by incubation at 45ºC for 15 minutes without agitation. The eppendorfs were centrifuged briefly and the supernatant was discarded. The cells were re-suspended in 1ml 1x TE pH 7.5. 200µL of transformation suspension was spread onto selective media.

2.2.8: Tetrad dissection

The strains that were subjected to tetrad dissection were in colonies of mixed mating type. These could not be used for back crossing as they would produce a false positive result, therefore the cells were streaked out for single colonies and grown for 3 days at 30ºC. Four single colonies, of unknown but heterothallic mating type, were selected and mated to 972 h-. The mating mixtures were microscopically inspected for the formation of asci. Asci could be differentiated from single cells as they were in a horse shoe or zig-zig shaped sac containing four dark spots. A small amount of mating mixture with plenty of asci (3 days old) was streaked across the bottom of a YES media plate. The asci were pulled out of the streak of cells using a tetrad dissector. This plate was left for an hour at 30ºC for the ascus to break down. Following this the spores were also separated with the tetrad dissector resulting in four separate colonies. This was incubated at 30ºC for 3 days. These individual colonies were then subjected to testing for aberrant invasive phenotypes.

2.2.9: Random spore analysis

The mating procedure was the same as for tetrad dissection. A 1µl inoculation loop-full of this mating mixture was added to 1ml distilled water. 10µL β-glucoronidase was added for overnight digestion. The digestion mixture was washed three times in distilled water via centrifugation at 5000rpm for 3 minutes. The number of cells in the washed mixture were counted using a haemocytometer. 200-500 spores were spread on YES media and incubated for 3 days at 30ºC. These individual colonies were then subjected to testing for aberrant invasive phenotypes.

2. 2.10: XGal assays

Surface colony overlay assay: Freshly grown cell suspensions of equal cell counts were spotted onto YES media. These were grown at 30ºC overnight. The following day the agarose overlay was prepared. 0.5g low-melt agarose was dissolved (with heat) in 93ml 0.5M potassium phosphate buffer. The following substances were then added to the solution under a fume hood: 6ml dimethylformamide (DMF) and 1ml 10% sodium dodecyl sulphate (SDS). The solution was left to cool. 1ml 100mg/ml Xgal (Melford) was added. 50µl of β-mercaptoethanol (BME) was added. This molten solution was poured directly onto colonies on the overnight growth plate. The plates were covered immediately to keep them in the dark. The overlay solution was left to set for 10 minutes. After 10 minutes the plates were transferred to the 30ºC incubator.
Invasive assay: LNB solid media was made as described above. 10µl of 100mg/ml Xgal for every ml of media made was added to the media whilst it was molten but cool. After this media had set and dried, strains were transferred for invasive growth.

2.3: Specialised methods

2.3.1: Comparison of the version β and version 2 libraries to identify strains in version 2 that were not in version β

The *S. pombe* Bioneer deletion libraries were provided with a reference spreadsheet that describes which gene is deleted in which strain. In these spread sheets the genes are named by a unique identifier. These unique identifiers were compared to identify which strains were in the version 2 library which were not in version β. The comparison was facilitated by Microsoft Access (http://office.microsoft.com/en-gb/access/), a database programme that can compare large tables of data and identify differences between them.

2.3.2: Resuscitation of the Bioneer *S. pombe* deletion library

The deletion libraries are stored in 96 well plates in 60% glycerol at -80ºC. Each 96 well plate has wells A-H, 1-12. To resuscitate the strains, the plates were removed from the freezer and briefly allowed to stand at room temperature until only the surface cells in each well started to thaw. The strains were transferred to yeast extract plus supplement (YES) media with an 8 by 6 pin stamp, allowing 48 strains per petri dish. A mark was made on the top, right-hand side of the petri dish external face and the cells were always transferred so that strains A1 or A7 were in the top, right-hand corner. Therefore, each of the 48 strains could be identified by its distance from these marker strains. Sterile techniques were used at all times. After the stamp had been used to transfer 48 cells, it was cleaned in a sonicating water bath (http://www.wolflabs.co.uk) for 30 seconds and then flamed in ethanol three times. It was then allowed to cool for 1 minute before it was used to transfer another 48 strains.

2.3.3: High-through put mating to convert the deletion libraries to prototrophy

The Bioneer deletion library is an auxotrophic collection of strains. Each strain has the genotype *h*+, ade-, leu-, ura-, ORF::KanR, where ORF is the specific open reading frame deleted in each strain. The Bioneer deletion library was converted to prototrophy by high through-put mating. The deletion library was haploid *h*+ mating type therefore 972 *h*- prototrophic strain was used. *S. pombe* strain 972 is the laboratory standard wild type. The auxotrophic deletion library was replicated and grown for 3 days on YES plates. 972 cell suspension was grown overnight in YES liquid medium. SPAS mating plates were prepared by spreading 120µL of 972 suspension across the surface. These were allowed to dry for 15 minutes prior to replica plating of the deletion library plates. The SPAS mating plates were then incubated at 27ºC for 3 days. After 3 days the colonies were inspected for asci. When plenty of asci, or spores, were seen, the mating strains were replica plated to YES +G418 selection plates. These were grown for 3 days at 30ºC. After 3 days the strains were replica plated to EMM plates for the second stage of selection.

2.3.4: Screening the deletion libraries for aberrant invasive phenotypes

After the second stage of selection, each strain was individually transferred to a YES + G418 agar plate to increase the colony size to enable a screen for aberrant invasive phenotypes. Eight strains were transferred to each plate. This required high attention to detail to ensure there was no cross contamination of strains. These plates were incubated for a further 3 days at 30ºC. After 3 days there were enough cells to set up the screen for aberrations in invasive phenotype. A large clump of cells was scraped together for each strain. The cells were taken from the YES + G418 plates with an inoculation loop. The clumps were transferred to LNB plates. Eight strains were transferred to each LNB plate. The strains were arranged in a circle to ensure there was no edge effect (Zupan et al. 2008). The LNB plates were left in the incubator for 7 days at 30ºC. After this time the strains were subjected to a invasive washing assay (Amoah-Buahin et al. 2005).

2.3.4i: Test for aberrant invasive and adhesive phenotypes

A gentle stream of water was washed over the clumps to determine whether or not the strains were adhesive. A more vigorous washing step followed which involved rubbing the surface cells off with a finger (Dodgson et al. 2009). Strains that had invaded the media left visible foci under the surface of the agar. After the washing stage all the strains were inspected under an Olympus CK2 bench-top light microscope and any aberrations in the morphology of efficiency of invasion were noted.

The routine concentration of agar in invasive media is 1% agar and this yields strong wild type *S. pombe* 972 invasion. To test the hyper-invasive strains, the agar concentration was increased to 2% which does not support invasive growth of wild type *S. pombe* 972.

2.3.5: BiNGO over-representation diagram calculations

The Benjamini and Hochberg false discovery rate (FDR) correction test is used to calculate the corrected P value for each GO term (Hochberg 1995). In the BiNGO diagrams GO terms are represented by circles that vary in colour and size, the circle colour is representative of the p value of significance and the size of the circle is representative of the number of genes attributed with that GO term.

2.3.6: Assay for highly invasive A1153 phenotype

Strains expressing GFP tagged proteins were created in the highly efficient invasive A1153 strain background to allow microscopy of actively growing filaments (Dodgson

et al. 2010). The presence of this phenotype within a strain is represented by "A1153" in the genotype. Strains with the GFP-tagged protein were crossed to an A1153 strain and the appropriate progeny were selected. The highly efficient invasive phenotype of A1153 is not a result of a single gene (Dodgson et al. 2010), therefore it cannot be tagged with a selectable marker. The highly efficient invasive phenotype is instead selected via an invasive assay. Toothpicks were used to transfer a small scrape of cells of the progeny from the A1153 cross to an SPAS plate. These plates were incubated at 30ºC for 3 days. The strains that have inherited the highly efficient invasive phenotype form extensive structures after just three days (Dodgson et al. 2010).

2.3.7: 8-Br-cAMP rescue experiments

Sterile filter paper discs were prepared with a 2cm diameter. These were then placed centrally on LNB media and soaked with 40µL of 0.1M 8-Br-cAMP (Amoah-Buahin et al. 2005). Strains were transferred at high cell density and placed 1cm away from the edge of the soaked filter disc. Eight strains were tested per plate. Strains were arranged in a circle. Plates were incubated for 7 days at 30ºC.

8-Br-cAMP is a lipophilic cAMP analogue that is more membrane permeant therefore easy for the cells to take up. Additionally it is *non-hydrolysable.* It mimics cAMP function and binds, thus activating PKA.

2.3.8: Preparation of strains for microscopy

Localisation of GFP tagged proteins was done in actively growing filaments in small glass- bottomed petri dishes (http://www.glass-bottom-dishes.com/). This meant the invasive structures did not need to be excised from the media for imaging. Images without GFP tagged proteins were taken of foci that had been excised from their growth media.

2.3.8i: Imaging of filaments

A small square of agar that contained invasive foci was excised from the growth plate with a scalpel. The excess agar was sliced off the bottom of the square. The invasive face of the square was placed on the glass microscope slide ready for microscopy (Dodgson et al. 2009).

2.3.8ii: Fluorescent imaging of filaments

All GFP localisations were done in actively growing filaments using small glass-bottomed dishes (http://www.glass-bottom-dishes.com/). These are small petri dishes which have a section of the plastic base replaced with a glass cover-slip. This allows high resolution images of actively growing filaments. The small glass bottom dishes were filled with agar and allowed to set. Once set, the plates were dried for 15 minutes in the 37ºC incubator with the lid off . Once dried, a sterile scalpel was used to make a small but deep incision in the agar. Cells were then transferred into the centre of this incision with an inoculation loop (Dodgson et al. 2010). Plates were incubated for 3 days prior to microscopic analysis. This method is only applicable to A1153 derived strains as it requires the highly invasive efficiency of this strain.

2.3.8iii: Fluorescent imaging of Cdc7-GFP in single cells

A streak of freshly growing Cdc7-GFP cells was used to inoculate 10ml of YES liquid media. This inoculum was grown overnight at 30ºC. The following morning 200µl of the overnight suspension was transferred to fresh growth medium for 4 hours prior to microscopic analysis. The fresh growth medium was made from 5ml YES, 5ml PEM buffer and 350µg Calcofluor. Calcofluor staining and light transmission methods were used for the differentiation between old and new cell end.

2.3.9: Microscopy

Localisation of GFP tagged proteins in filaments used a Zeiss LSM510 Meta confocal microscope. Images were captured with the transmitted light of either the Zeiss LSM510 Meta confocal microscope or the Zeiss axiovert 200M microscope. 40x water immersion, 60x and 100x oil immersion lenses were used.

2.3.9i:Visualisation of fluorescence in filaments

An 488nm argon laser was used for excitation. The primary dichroic (HFT UV/488/543/633) was set to focus the 488nm excitation beam onto the sample. This same dichroic only lets light above 488nm back through, which is subsequently focused towards the collection filter. A BP 505-530 collection filter was used. GFP images were captured with a 100x oil immersion lens.

2.3.9ii: Visualisation of fluorescence in single cells

GFP fluorescence was visualised as described above. The wide-field set up of the Zeiss Axiovert 200M microscope was used for visualisation of Calcofluor staining and Cdc7-GFP localisation for the Cdc7-GFP cells only. A FITC filter was used for GFP fluorescence and UV filter for Calcofluor fluorescence.

2.3.9iii: Time lapse

A Zeiss LSM510 Meta confocal microscope scanned a 90µm square section of actively growing filaments every 5 minutes over 6.5 hours. The microscope collected multiple images over the vertical axis to ensure the correct focal plane was captured. The GFP set up described above was used. To ensure the filaments continued to grow once removed from the incubator a temperature chamber set to 30ºC was used during the microscopy.

2.3.9iv: Image processing

All images were processed in Adobe Photoshop version 7 (http://www.photoshop.com/) or Image J (http://rsbweb.nih.gov/ij/index.html).

2.4: Strains and plasmids

2.4.1: The Bioneer deletion library

The Bioneer deletion library is an auxotrophic collection of strains. Each strain has the genotype h+, ade-, leu-, ura-, ORF::KanR, where ORF is the specific open reading frame deleted in each strain. This study produced a prototrophic version of the libraries which had the genotype ade+, leu+, ura+, ORF::KanR.

2.4.2: Table of strains

Strain	Source	Genotype
972	Laboratory stock	h- ade+, leu+, ura+
556	Laboratory stock	h+, ade-, leu-, ura-
A1153, leu-,	James Dodgson	A1153, leu-, Lys:KanR
FWP77	Charlie Hoffman	leu1-, ura4::fbp1-lacZ
Fbp1, *fep1Δ*	This thesis	leu+, ade+, ura4::fbp-lacZ, *fep1Δ*::KanR

Fbp1, *imp1Δ*	This thesis	leu+, ade+, ura4::fbp-lacZ, *imp1Δ*::KanR
Fbp1 , *pob3Δ*	This thesis	h?, leu+, ade+, ura4::fbp-lacZ, *pob3Δ*::KanR
Fbp1, *git31Δ*	This thesis	leu+, ade+, ura4::fbp-lacZ, *git3Δ*::KanR
Fbp1, *spac664.03Δ*	This thesis	leu+, ade+, ura4::fbp-lacZ, *spac664.03Δ*::KanR
Alg10-GFP	This thesis	ade+, ura+, leu::Alg10-GFP-YFP-FLAG-His, A1153 NB. Created with Riken pDUAL plasmid (see below)
Spcc18.10-GFP	This thesis	ade+, ura+, leu::Spcc18.10-GFP-YFP-FLAG-His, A1153 NB. Created with Riken pDUAL plasmid (see below)
Cdc7-GFP	James Dodgson	Cdc7-GFP-ura4+, ura-, ade+ leu+, A1153 NB. This strain was created from Cdc7-GFP-ura4+ strain (M. Sohrmann, 1998.)
Cdc7-GFP, *pom1Δ*	This Thesis	Cdc7-GFP-ura4+, ura-, ade+ leu+, A1153, *pom1*::KanR
Cdc7-GFP, *tea1Δ*	This Thesis	Cdc7-GFP-ura4+, ura-, ade+ leu+, A1153, *tea1*::KanR
Cdc7-GFP, *tip1Δ*	This Thesis	Cdc7-GFP-ura4+, ura-, ade+ leu+, A1153, *tip1*::KanR
Cdc7-GFP, *tea2Δ*	This Thesis	Cdc7-GFP-ura4+, ura-, ade+ leu+, A1153, *tea2*::KanR
Cdc7-GFP, *tea4Δ*	This Thesis	Cdc7-GFP-ura4+, ura-, ade+ leu+, A1153, *tea4*::KanR
Cdc7-GFP, *fin1Δ*	This Thesis	Cdc7-GFP-ura4+, ura-, ade+ leu+, A1153, *fin1*::KanR

YDM1060	Dannel McCollum	h+, Spg1-GFP-KanR, leu-, ura-
Spg1-GFP	This thesis	Spg1-GFP:KanR, A1153
IH2673	Iain Hagan	Fin1.4GFP-KanR, ura-
Fin1-GFP	This thesis	Fin1.4GFP-KanR, A1153

2.4.3: Creation of novel strains

Fwp77, ORFΔ strains: FWP77 was mated to each ORF:KanR, ade+, leu+, ura+ strain individually. The appropriate progeny were selected by growth on YES + G418 followed by secondary selection on EMM media +uracil + 5FOA. 5FOA allows selection of Ura- strains.

Alg10-GFP, Spcc18.10-GFP: These strains were created with the pDUAL expression clones. These were purchased from Riken BRC DNA bank (http://dna.brc.riken.jp/). The expression clones were stored in Dh5α competent *E. coli* cells therefore they initially had to be extracted from these cells. The extracted plasmid was then linearised with Not1 restriction enzyme. This was then used for the transformation of A1153, leu- and the successful transformants are selectable by leucine prototrophy (Matsuyama et al. 2004).

Cdc7-GFP, *ORFΔ:* Cdc7-GFP was mated to each ORF:KanR, ade-, leu-, ura- strain individually. The appropriate progeny were selected by growth on YES + G418 followed by secondary selection on EMM media and finally an invasive assay to select the strains had inherited the A1153 highly invasive phenotype.

Fin1-GFP: IH2673 was mated to A1153. The appropriate progeny were selected on YES +G418 then subjected to an invasive assay to identify A1153 phenotype strains.

Spg1-GFP: YDM1060 was mated to A1153. The appropriate progeny were selected and subjected to an invasive assay to identify A1153 phenotype strains.

2.4.4: Plasmids

Plasmid Name	Source	Use
pON177	Created by Olaif Nelson, (http://pombe.kaist.ac.kr/nbtsupp/protocols/gen_anal_haploid.php)	Transformation of *Spbc1289.15*Δ (Chapter 3, section 3.2.6)
pDUAL, Alg10-YFP-FLAG-His	Riken DNA Bank (http://dna.brc.riken.jp/)	Transformation of A1153 leu –, (Chapter 5, section

tag expression clone		5.2.8)
pDUAL, Spcc18.10-YFP-FLAG-His tag expression clone	Riken DNA Bank (http://dna.brc.riken.jp/)	Transformation of A1153 leu –, (Chapter 5, section 5.2.8)

Chapter 3: A screen for *S. pombe* strains aberrant in the low nitrogen induced invasive process

3.1: Aims of the screen

S. pombe is capable of invasive growth on low-nitrogen base (LNB) (Amoah-Buahin et al. 2005) and rich (YES) (Dodgson et al. 2009) media. Induction of invasion is thought to differ on these two media types. Previous investigation of the invasive process involved a screen of the *S. pombe* Bioneer deletion library for non-invasive strains on YES (Dodgson et al. 2009). This screen identified genes essential for invasion, but did not identify any genes involved in the response to low nitrogen. Following this, Dodgson *et. al*. (2009) described morphological aberrations of five strains (*tea1Δ, tea2Δ, tip1Δ, mal3Δ* and *tea4Δ,*) that were individually selected for analysis due to their roles in growth polarity. These strains invaded the media with an efficiency equal to *S. pombe* 972 but exhibited an aberrant invasive morphology, which raised the questions, how many other such strains exist and what morphologies may they exhibit?

These questions are addressed here by performing a screen of the *S. pombe* deletion library for strains aberrant in the invasive process (from here on referred to as "the screen"). Additionally, the screen was performed on LNB to identify any potential nitrogen response associated genes that would not have been identified from the screen on YES.

3.2: Results

The Bioneer corporation have produced three versions of the *S. pombe* deletion library, version β, version 1 and version 2 (http://pombe.bioneer.co.kr/). Each library produced contains more strains than the previous version. The deletion libraries are supplied with a reference spread sheet that identifies the deleted gene in each strain. Version β and version 2 were used in this body of work. Initially only version β was available for study therefore a complete screen of version β was performed; following this version 2 became available.

A comparison of the version β and version 2 reference spread sheets (Materials and Methods) identified 654 strains that were in version 2 but not in version β. These were individually selected from the version 2 library and subjected to the same screening procedure as the version β strains. The results presented here are from the initial version β screen and the subsequent screen of the 654 version 2 strains.

The first stage of the screen was converting the strains to an appropriate nutritional background.

3.2.1: Conversion to prototrophy does not alter invasive phenotype

The Bioneer *S. pombe* deletion libraries are collections of auxotrophic strains that require the addition of nutritional supplements to media for growth. These supplements provide a significant source of nitrogen therefore preventing the identification of genes specifically required for the response to low nitrogen. Converting the library to prototrophy eliminates this problem, as nutritional supplements no longer need to be added.

High through-put mating methods (Material and Methods) were used to convert the Bioneer *S. pombe* deletion libraries to prototrophy. To determine whether the conversion of the libraries to prototrophy affected the invasive phenotype, ten strains were tested for differences in invasive efficiency between the prototrophic and auxotrophic genotype. Equal invasive efficiency was exhibited by all strains tested (figure 3.1). The two strains shown in figure 3.1 are a representative of the ten strains tested (table 3.1).

Strain	Invasive phenotype of prototroph on YES	Invasive phenotype of auxotroph on YES
972	+	N/A
556	N/A	+
tea1Δ	+	+
tea2Δ	+	+
tip1Δ	+	+
spcc18.10cΔ	+	+
alg10Δ	+	+
ral2Δ	-	-
ivn1Δ	-	-
adn2Δ	-	-
for3Δ	-	-
adn1Δ	-	-

Table 3.1 The invasive efficiency displayed by a selection of strains as prototrophs and auxotrophs for adenine, leucine and uracil on YES. +: normal invasion as seen for *S. pombe* strain 972. -: no invasion.

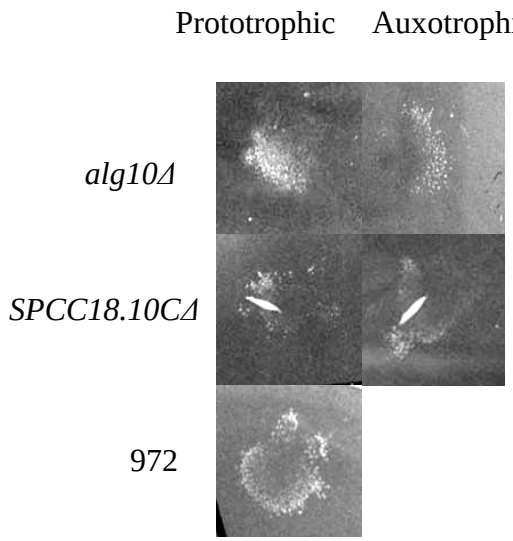


Figure 3.1 The effect of prototrophy on invasive efficiency. The invasive efficiency of two prototrophic (ade+ leu+, ura+) strains (*alg10Δ* and *spcc18.10cΔ*) were compared to the auxotrophic (ade-, leu-, ura-) counterparts. After washing no invasive difference was noted between the two genotypes.

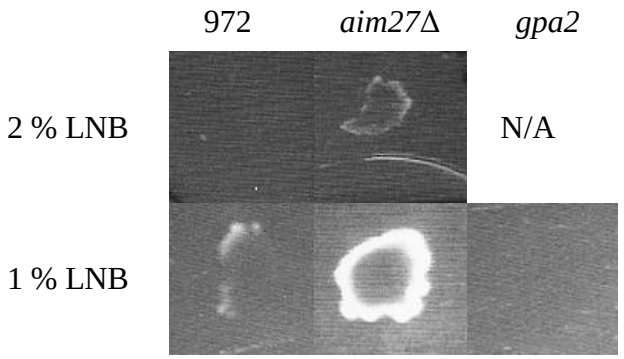


Figure 3.2 Invasive efficiency defects displayed by non-invasive (*gpa2Δ)* and hyper-invasive (*aim17Δ)* strains. 972 is non-invasive on 2% LNB but invasive on 1% LNB. Hyper-invasive strain *aim27Δ* can invade on both 1% and 2% LNB, whereas *gpa2Δ* is non-invasive on 1% LNB.

The conversion of the deletion libraries to prototrophy will affect the number of strains tested for aberrations in the invasive growth process.

3.2.2: 3082 strains were tested for aberrations in the invasive growth process

Prototrophic growth and converting the libraries to prototrophy involves multiple cellular processes which will require genes represented in the deletion libraries. Therefore, the strains that have these genes deleted could not be recovered as prototrophic strains. This affected the total number of strains tested for aberrations in the invasive process. Version β (2638 strains) was converted to prototrophy. Following this version 2 was converted to prototrophy and the 654 additional strains of version 2 were individually selected. There were 210 strains (appendix 3.3) that could not be recovered from the prototrophic conversion of version β and the 654 additional version 2 strains.

Therefore, the number of strains available for the screen was 3082;

	2638	(version β)
+	654	(version 2)
–	210	(no prototrophic isolates)

Prior to the screen of these 3082 strains for aberrations in the invasive process, the preparation of media had to be optimised.

3.2.3: Media must contain 1% agar and set in covered conditions

Invasive growth by *S. pombe* requires solid media, which is made with agar. The agar constituent, as well as the method used for media production is extremely important. In the initial stages of this work the agar used came from a finite source that was exhausted during the preparation of this thesis therefore an alternative agar source was used; NZ agar (B.T.P Drewitt, London). Unfortunately the NZ agar did not yield consistently strong invasion when prepared using the previous method so various alternative agars were tried but none were effective. The routine agar concentration used in media preparation is 2% (20 g/l). Converting this to 1% (10 g/l) resulted in consistently strong invasion on NZ agar media (figure 3.2).

The media preparation method also affects invasive efficiency. Preparation of invasive media involves five stages; melting agar, mixing constituents, pouring plates, setting plates and finally, drying plates. Though all five stages are common to general

media preparation, it was found that the method for setting agar plates is important in preparation of media for invasive growth. When plates are left to set, they must be covered. Wild type *S. pombe* 972 (referred to as *S. pombe* 972 from here) shows significantly poorer invasive efficiency on plates that have been left to set uncovered.

The optimization of media preparation allowed the screen of the Bioneer *S. pombe* deletion libraries for strains aberrant in the low-nitrogen-induced invasive process. Inspection of the media for aberrations in invasive efficiency was followed by microscopic observation for aberrations (Materials and Methods). Strains that showed a preliminary phenotype were retested a minimum of four times before being attributed with a mutant phenotype. This identified three phenotypic groups that were aberrant in invasive efficiency.

3.2.4: Strains that were aberrant in invasive efficiency

Inspection of the media for aberrations in invasive efficiency identified three phenotypes: non-invasive, poorly invasive and hyper-invasive (figure 3.2). Adhesion precedes invasion during the invasive process (Dodgson et al. 2009) therefore non-invasive and poorly invasive strains were also subjected to adhesion tests. Those exhibiting defects were classified as non-adhesive. Additionally, *par1Δ* was identified in the screen as potentially hyper-adhesive, so this was also tested.

3.2.4i: 11 strains were non-adhesive

11 strains were attributed with a non-adhesive and one with a hyper-adhesive phenotype on LNB (figure 3.3, table 3.2).

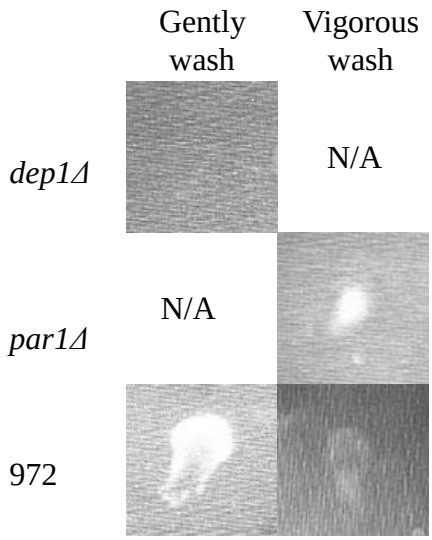


Figure 3.3 Adhesion defective strains. After a gentle wash, all surface cells from non-adhesive (*dep1Δ)* strains are rinsed away, where-as hyper-adhesive *par1Δ* remains firmly attached to the agar even after a vigorous wash.

Strain	**Adhesion on LNB**	**Strain**	**Adhesion on LNB**
atg14Δ	-	*tal1Δ*	-
fta5Δ	-	*adn3Δ*	-
spbc16g5.02cΔ	-	*adn2Δ*	-
hsr1Δ	-	*snf5Δ*	-
sol1Δ	-	*pyp1Δ*	-
dep1Δ	-	*par1Δ*	++
972	+		

Table 3.2 Strains exhibiting adhesive defects on LNB. +: adhesion as seen for *S. pombe* 972, -:non-adhesive, ++: hyper-adhesive.

The strains that could adhere to the media were then further characterised as non-invasive or poorly invasive.

3.2.4ii: 19 strains were non-invasive

19 strains, including 10 which were non-adhesive, were characterised as non-invasive (figure 3.2, table 3.3). Eight of the 19 are nuclear based proteins with roles such as transcription factors, karyopherins and chromatin remodelling. Three of the 19 do not have orthologues, nor have they been experimentally characterised. Git3 and Gpa2 are the G protein linked receptor and α subunit of the glucose activated cAMP-Pka signalling cascade respectively (Welton et al. 2000). Ogm1 is involved with cell surface glycoprotein modification (Willer et al. 2005) and the *S.cerevisiae* Ccr1 orthologue NCP1 is involved in ergosterol synthesis (Lin et al. 2009), a major cell membrane component. Efc25 is a putative GEF for Ras1, which is part of the cell morphology signalling cascade (Tratner et al. 1997). The 19 non-invasive strains represent genes attributed with a wide variety of gene ontology (GO) terms. Bio-informatics platforms, such as Cytoscape, facilitate analysis of these GO terms which may highlight cellular events that take place during the invasive process.

Cytoscape facilitates visual analysis of gene ontology (GO) terms and their relationships (Shannon et al. 2003). BiNGO is a Cytoscape plug-in that analyses a set of

Gene ID	Phenotype	Gene Name	Gene Description
SPBC336.03	Non-invasive	*efc25*	GEF for Ras1-Scd1 mating MAPK pathway
SPAC2F7.08c	Non-invasive	*snf5*	SWI/SNF complex subunit, involved in chromatin remodelling(predicted).
SPBC30B4.04c	Non-invasive	*sol1*	SWI/SNF complex subunit, involved in chromatin remodelling.
SPAC23H3.13c	Non-invasive	*gpa2*	G-protein alpha subunit for glucose driven cAMP signalling.
SPBC609.05	Non-invasive	*pob3*	FACT complex component (predicted) involved in chromatin organization.
SPBC21C3.02c	Non-invasive	*dep1*	Histone deacetylation, involved in chromatin remodelling.
SPBC1289.10c	Non-invasive	*adn2*	Transcriptional regulator, positively regulates cell-substrate adhesion.
SPCC1494.10	Non-invasive	*adn3*	Transcriptional regulator, positively regulates cell-substrate adhesion.
SPCC1753.02c	Non-invasive	*git3*	Glucose-triggered adenylate cyclase 7TM activation Gprotein
SPAC22A12.07c	Non-invasive	*ogm1*	Protein O-mannosyltransferase, cell wall mannoprotein biosynthesis.
SPAC4G8.03c	Non-invasive		RNA-binding protein.
SPAC2G11.03c	Non-invasive	*vps45*	Vacuolar sorting protein Vps45, involved in vesicle docking in cytokinesis.
SPAC8E11.05c	Non-invasive		Conserved fungal protein.
SPAC3H1.11	Non-invasive	*hsr1*	Transcription factor Hsr1, involved in oxidative stress response.
SPAC1F8.06	Non-invasive	*fta5*	Cell surface glycoprotein with GLEYA domain, involved in cell adhesion.
SPBC18H10.19	Non-invasive	*atg14*	PI-3-kinase subunit, involved in macroautophagy and CVT pathway.
SPBC29A10.01	Non-invasive	*ccr1*	NADPH-cytochrome p450 reductase, involved in ergosterol biosynthesis.

Table 3.3

Gene ID	Phenotype	Gene Name	Gene Description
SPAC18G6.13	Non-invasive		Sequence orphan, localises to nuclear envelope.
SPBC16G5.02c	Non-invasive		Ribokinase.

Table 3.3 Non-invasive strains identified from version β and version 2 Bioneer *S. pombe* deletion library screen on LNB. All gene descriptions are taken from Gene DB (http://old.genedb.org/genedb/pombe/ last accessed 11/07/2011)

genes for GO term over-representation in comparison to a reference gene set (Maere et al. 2005). In this study the reference gene set used was the entire genome. The non-invasive group of genes was subjected to BiNGO analysis (figure 3.4a).

The non-invasive BiNGO diagram suggests cell adhesion is over represented within the non-invasive genes. Fta5 and Ogm1are attributed with this GO term. Fta5 is a cell surface glycoprotein, with a GLEYA domain (Linder et al. 2008). Ogm1 is involved in cellular conjugation and mating (Tanaka et al. 2005). The chromatin modification GO term is representative of *pob3*, *snf5*, *sol1* and *dep1*.

The non-invasive strains exhibit a severe block during the invasive process which results in a lack of invasion. A less severe exhibition of this phenotype is present in the strains classified as poorly invasive.

3.2.4iii: 15 strains were poorly-invasive

15 strains exhibited a poorly invasive phenotype (table 3.4). Seven of these strains represent proteins that have nuclear based role such as transcriptional regulators and chromatin re-modellers. For3 is involved in actin cable assembly (Wang et al. 2008) and Git5 is the β subunit of the G protein activated by glucose (Welton et al. 2000). There are two RNA associated proteins within this group and an autophagy associated protein, Atg9. Met14 is an adenyl sulphate kinase and Spbpb7E8.02 is an uncharacterised protein (http://old.genedb.org/genedb/pombe/). Finally Pyp1 is a protein phosphatase in the stress-activated MAPK pathway (Dal Santo et al. 1996).

These strains were also subjected to BiNGO analysis (figure 3.4b, appendix 3.2). Nuclear based GO terms are represented on this BiNGO diagram, indicating the nucleus as the main region for regulation of invasive efficiency.

The non- invasive and poorly-invasive phenotypic groups represent genes that may be involved in positive regulation of the invasive process. The final group exhibiting aberrations in invasive efficiency are the hyper-invasive strains. These represent potential negative regulators of the invasive response. The rest of the phenotypic groups to be presented did not contain enough strains for a BiNGO analysis or the BiNGO analysis did not provide any over-represented GO terms.

3.2.4iv: 9 strains were hyper-invasive

The routine concentration used for invasive media is 1% agar and this yields strong invasion of *S. pombe* 972. To determine whether or not a strain was hyper-invasive,

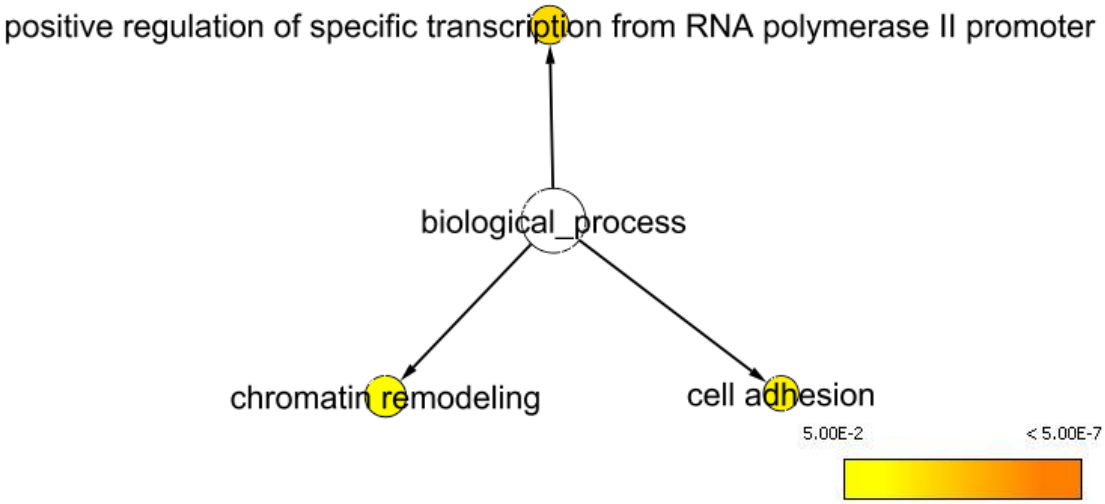


Figure 3.4a BiNGO over-representation diagram for the non-invasive strains. The colour of the circle represents the significance of over-representation which is given on a scale in the bar on the right.

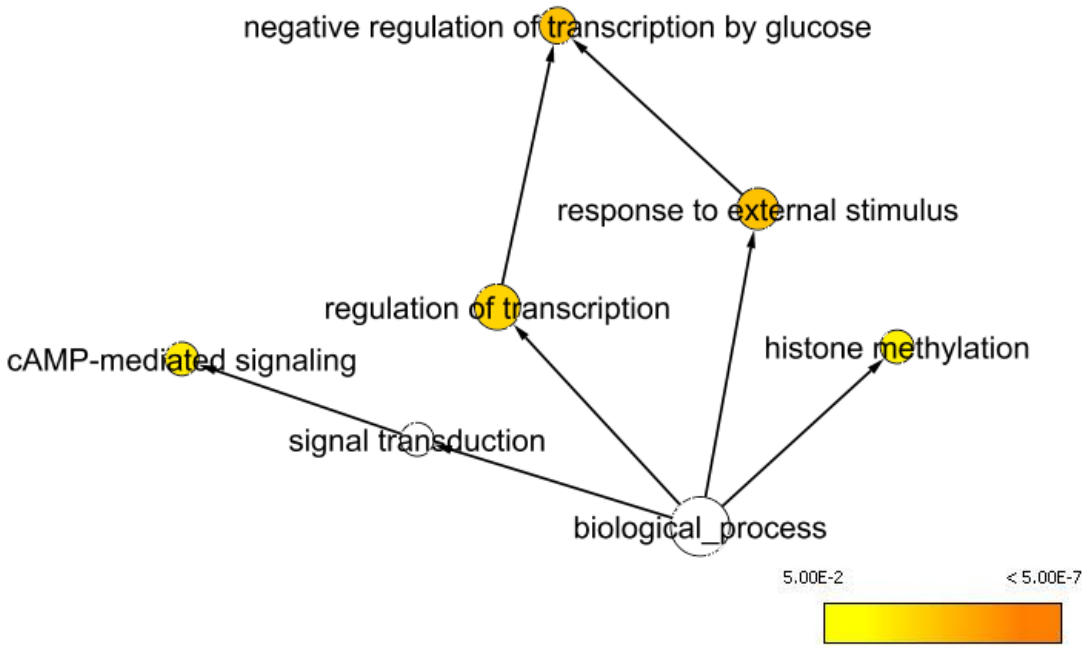


Figure 3.4b BiNGO over-representation diagram for poorly- invasive strains. The colour of the circle represents the significance of over-representation which is given on a scale in the bar on the right.

Gene ID	Phenotype	Gene Name	Gene Description
SPBC530.08	Poorly-Invasive		Transcriptional regulator (predicted).
SPBC30B4.03c	Poorly-Invasive	*adn1*	Adhesion defective protein, predicted transcriptional regulator.
SPBC18H10.06c	Poorly-Invasive	*swd2*	SET1 complex subunit, involved in chromatin silencing and histone methylation.
SPAC32A11.03c	Poorly-Invasive	*phx1*	Transcription factor.
SPAC1782.11	Poorly-Invasive	*met14*	Adenylylsulfate kinase (predicted), involved in sulphate assimilation.
SPCC895.05	Poorly-Invasive	*for3*	Formin, involved in actin cable bundle assembly.
SPCC1223.13	Poorly-Invasive	*cbf12*	Transcription factor, involved in cell-cell adhesion and cytokinesis.
SPBC1604.08c	Poorly-Invasive	*imp1*	Karyopherin (predicted).
SPAC23E2.01	Poorly-Invasive	*fep1*	Iron-sensing transcription regulator, involved in iron transporter gene expression.
SPBC32H8.07	Poorly-Invasive	*git5*	G-protein beta subunit for glucose driven cAMP increase.
SPAC26F1.10c	Poorly-Invasive	*pyp1*	Phosphoprotein phosphatase, de-phosphorylates Sty1 MAP kinase.
SPBPB7E8.02	Poorly-Invasive		Conserved protein (fungal bacterial protazoan).
SPAC6G9.14	Poorly-Invasive		RNA-binding protein.
SPBC15D4.07c	Poorly-Invasive	*Atg9*	Autophagy associated protein.
SPAC664.03	Poorly-Invasive		RNA polymerase II associated Paf1 complex.

Table 3.4 Poorly invasive strains identified from version β and version 2 Bioneer *S. pombe* deletion library screen on LNB. All gene descriptions are taken from Gene DB (http://old.genedb.org/genedb/pombe/)

strains were grown on 2% LNB. This prevents invasion of *S. pombe* 972 but not that of the hyper-invasive strains (figure 3.2). There are nine strains categorized as hyper-invasive (table 3.5).

Cnb1Δ represent the proposed regulatory subunit of calcineurin, a protein phosphatase (Sio et al. 2005). Two strains (*spbc1198.06cΔ* and *wsc1Δ*) represent proteins that are involved in cell wall biosynthesis (http://old.genedb.org/genedb/pombe/). *Rga8* encodes a Rho1 GTPase activating protein (GAP) (Yang et al. 2003) and *rcd1* encodes a protein involved in signal transduction of the mating response (Okazaki et al. 1998).

Ace2Δ represents a transcription factor responsible for up-regulating the expression of genes involved in cell separation (Alonso-Nunez et al. 2005). *Rps801* represents a ribosomal protein, *aim27* represents an ER membrane component subunit and *sgo1*represents an inner centromere protein (http://old.genedb.org/genedb/pombe/). The identification of these nine hyper-invasive strains concludes the description of strains aberrant in invasive efficiency. The invasive morphology of the prototrophic *S. pombe* Bioneer deletion libraries was then analysed. This allowed the identification of four novel morphological mutant phenotypes.

3.2.5: Strains with aberrant morphologies were identified in the screen

The morphology of *S .pombe* invasive growth has been previously described (Amoah-Buahin et al. 2005; Dodgson et al. 2009; Dodgson et al. 2010). Invasive foci form as a central mass of cells with filamentous protrusions radiating out from them. The filaments are made from cells which are usually parallel-sided and uniform in size as well as morphology (figure 3.5). The filaments then grow around each other forming rope structures.

The LNB screen allowed identification of novel aberrant phenotypes. Strains that exhibit aberrant morphologies could be separated into two classes (I) those that exhibited aberrations in the rope structures of the filamentous protrusions and (II) those that exhibited aberrations in the single cells of the filamentous protrusions. These two classes could then be further subdivided in two: (Ia) **Strains that were unable to form filamentous protrusions** (figure3.5, table 3.6). Strains were able to invade and form central foci but unable to form filamentous rope structures radiating out from them. (Ib) **Strains that had thickened filamentous rope structures** (figure 3.5, table 3.7). Strains had more cells forming the rope structures of the filamentous protrusions (arrow in figure 3.6 class Ib), giving a thickened appearance. (IIa) **Strains that had elongated**

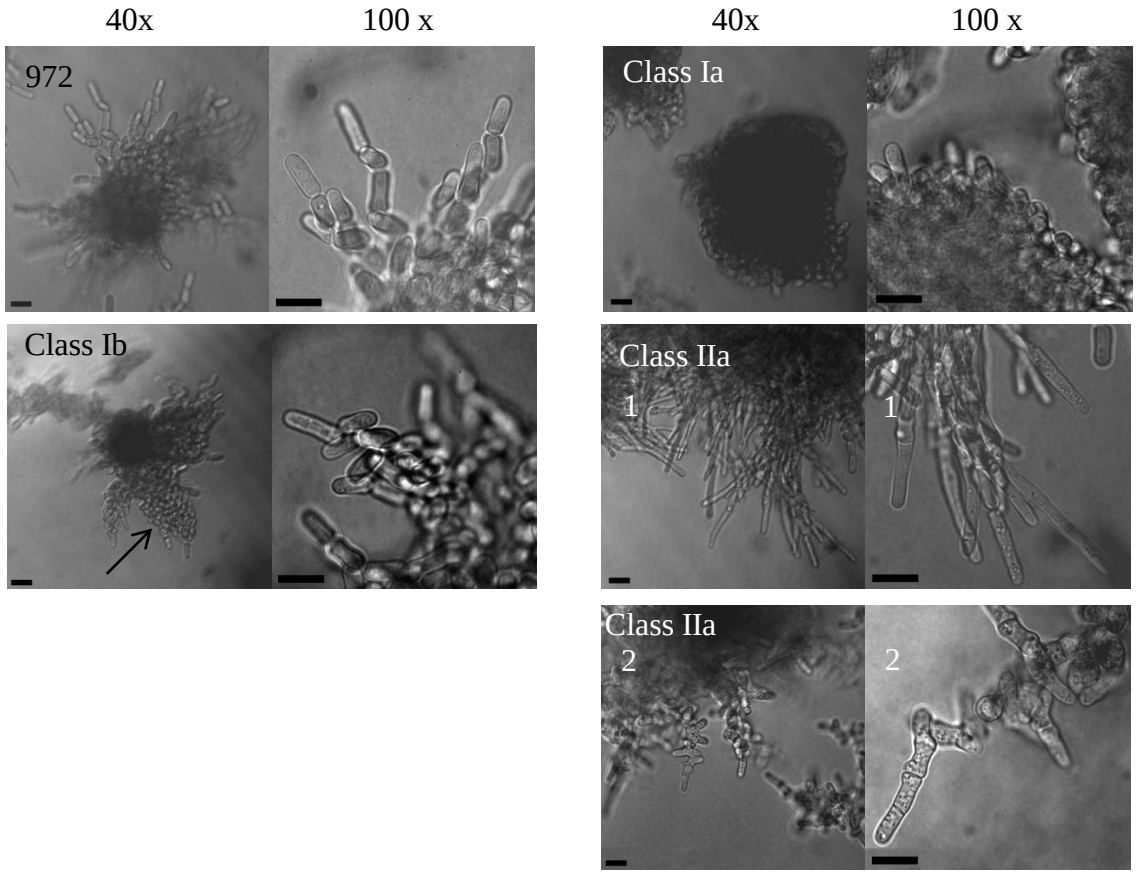


Figure 3.5. Morphological classes identified from the screen. Class Ia) Were unable to form filamentous protrusions. 40x: *fkh2Δ* 100x: *fkh2Δ* Class Ib) Had thickened filamentous rope structures. 40x:*acp1Δ* arrow points to thickened rope structure.100x: *fhn1Δ* Class IIa) Had elongated filamentous cells. 1:40x: *sty1Δ* 100x: *sty1Δ* 2: *sep1 Δ*. The morphology of Sep1 differs to that of other class IIa strains

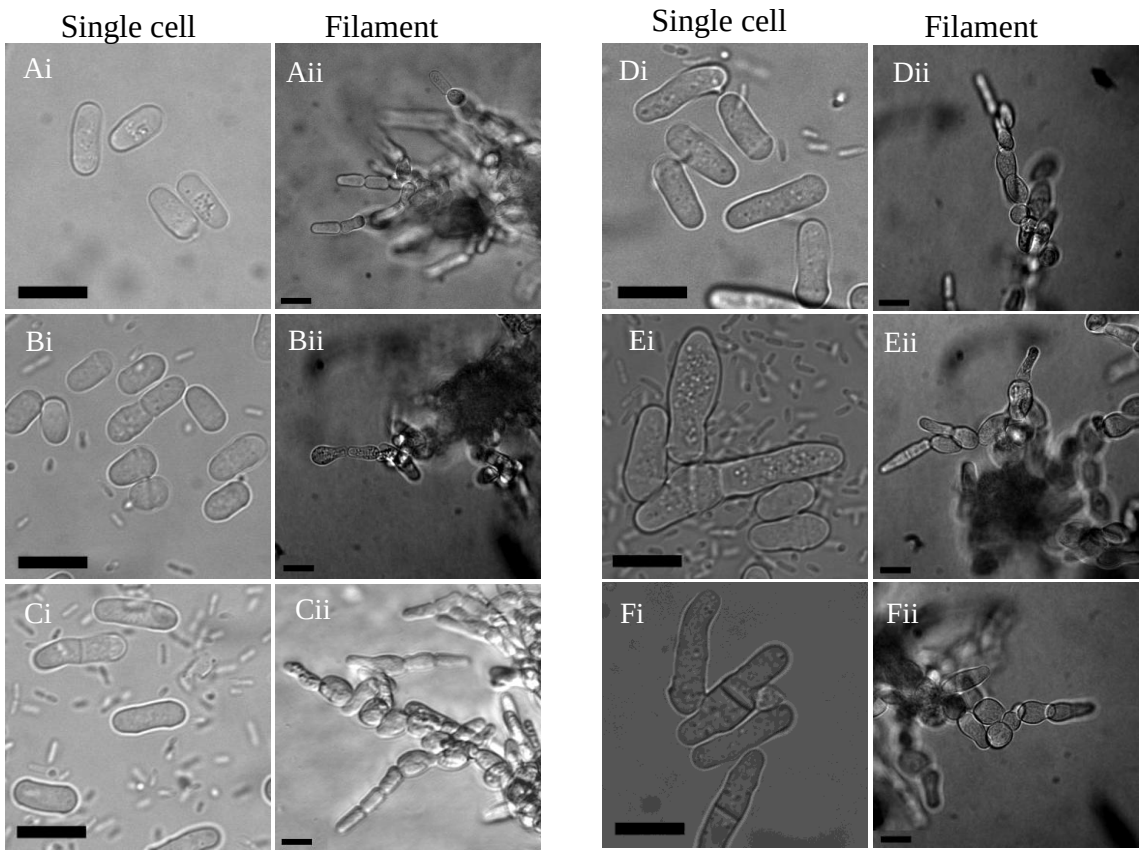


Figure 3.6. Class IIb strains that exhibited erratically structured filamentous cells. Invasive filamentous strains grown on LNB for 7 days. Single cells grown over night in LNB. Strains exhibit varying degrees of mutant morphology as single cells. i) single cell ii)filament. A) 972 B) *rpn10Δ* C) *gsa1Δ* D) *sep11*Δ E) *SPBC19G7.10cΔ* F) *cti1Δ*. Bar 10µM.

Gene ID	Phenotype	Gene Name	Gene Description
SPCC830.06	Hyper-invasive	*cnb1*	Predicted calcineurin b, regulatory subunit.
SPBC1198.06c	Hyper-invasive		Mannan endo-1,6-alpha-mannosidase involved in fungal type cell wall biogenesis.
SPAC13A11.01c	Hyper-invasive	*rga8*	GTPase activating protein for target Rho1 (implicated).
SPAC6G10.12c	Hyper-invasive	*ace2*	Transcription factor.
SPAC29B12.06c	Hyper-invasive	*rcd1*	RNA-binding protein, involved in nitrogen induced sexual development.
SPAC2C4.16c	Hyper-invasive	*rps801*	40S ribosomal protein S8.
SPBC1711.03	Hyper-invasive	*aim27*	ER membrane protein complex subunit.
SPBP35G2.03c	Hyper-invasive	*sgo1*	Inner centromere protein, involved in meiotic sister chromatid cohesion.
SPBC30B4.01c	Hyper-invasive	*wsc1*	Transmembrane receptor Wsc1, involved in actin cytoskeleton organization.

Table 3.5 Hyper-invasive strains identified from version β and version 2 Bioneer *S. pombe* deletion library screen on LNB. All gene descriptions are taken from Gene DB (http://old.genedb.org/genedb/pombe/)

Gene ID	Phenotype	Gene Name	Severity of Phenotype	Gene Description
SPCC417.07c	Class Ia	*mbo1*	+++	Microtubule organiser centre.
SPCC18.10	Class Ia	*spcc18.10*	+	Pyridoxine-pyridoxal-pyridoxamine kinase.
SPBC1604.20c	Class Ia	*tea2*	+++	Kinesin-like protein, involved in localization of polarity factors
SPCC1223.06	Class Ia	*tea1*	+++	Involved in establishment and maintenance of cell polarity.
SPAC56F8.06c	Class Ia	*alg10*	++	Involved in N-linked glycosylation.
SPAC3C7.12	Class Ia	*tip1*	+++	Involved in microtubule stabilization and targeting to the cell ends.
SPAC13G6.10c	Class Ia	*asl1*	++	Cell wall protein, O-glucosyl hydrolase.
SPBC1271.12	Class Ia	*kes1*	++	Oxysterol binding protein, involved in ergosterol biosynthesis.
SPBC16G5.15c	Class Ia	*fkh2*	++	Fork head transcription factor, involved in contraction of the division septum .

Table 3.6 Class Ia morphological mutant strains identified from version β and version 2 Bioneer *S. pombe* deletion library screen on LNB. All gene descriptions are taken from Gene DB (http://old.genedb.org/genedb/pombe/)

Gene ID	Morphology	Gene Name	Gene Description
SPAC56F8.02	Class Ib		AMP binding enzyme (predicted)
SPAC823.05c	Class Ib	*tlg2*	Involved in intracellular protein transport
SPBC2G2.07c	Class Ib	*mug178*	Mitochondrial ribosomal protein
SPBC947.15c	Class Ib		NADH dehydrogenase
SPBP8B7.13	Class Ib	*vac7*	Positive regulation of protein kinase activity
SPBC1734.08	Class Ib	*hse1*	Involved in ascospore-type prospore formation
SPAC17G8.05	Class Ib	*med20*	Involved in cellular protein localization
SPAC31G5.11	Class Ib	*pac2*	cAMP-independent regulatory protein, involved in sexual development
SPCC576.13	Class Ib	*swc5*	Chromatin remodelling complex
SPAC2F7.09c	Class Ib		Mitochondrial GTPase related protein
SPAC222.05c	Class Ib	*mss1*	Mitochondrial GTPase, involved in mitochondrial translation
SPBC354.15	Class Ib	*fap1*	Fructosyl amino acid oxidase (predicted), involved in L-lysine biosynthesis
SPBC25B2.04c	Class Ib	*mtg1*	Mitochondrial GTPase involved in translation
SPBC16G5.16	Class Ib		Transcriptional regulator (predicted), zinc finger protein
SPAC23C4.12	Class Ib	*hhp2*	Serine/threonine protein kinase, involved in DNA repair
SPAC1093.03	Class Ib		Polyphosphoinositide phosphatase activity

Table 3.7

Gene ID	Morphology	Gene Name	Gene Description
SPBC1706.01	Class Ib	*tea4*	Involved in cell polarity
SPBC3B8.02	Class Ib	*php5*	Transcription factor, involved in response to nutrients
SPAC18G6.15	Class Ib	*mal3*	Involved in microtubule integrity
SPAC9.12c	Class Ib	*atp12*	F1 ATPase chaperone (ISS), involved in mitochondrial F1-F0 ATPase assembly
SPAC1610.02c	Class Ib		Mitochondrial ribosomal protein l1
SPBC4F6.08c	Class Ib	*mrpl39*	Mitochondrial ribosomal protein subunit l39
SPBC19F8.08	Class Ib	*rps401*	40S ribosomal protein
SPAC25B8.05	Class Ib		t-RNA pseudouridylate synthase (predicted)
SPBC30B4.03c	Class Ib	*adn1*	Adhesion defective protein, predicted transcriptional regulator
SPCC338.14	Class Ib		Adenosine kinase (predicted)
SPBC543.07	Class Ib	*pek1*	Serine/threonine protein kinase, Mkh1p-Pek1p-Spm1p MAP kinase pathway
SPCC1672.03c	Class Ib		Guanine deaminase (predicted)
SPBC16A3.01	Class Ib	*spn3*	Septin, involved in cytokinetic cell division
SPBC119.12	Class Ib		Involved in intracellular protein transport
SPAC12B10.07	Class Ib	*acp1*	F-actin capping protein (alpha subunit), involved in actin filament organization
SPAC22H10.03c	Class Ib	*kap114*	Karyopherin, involved in intracellular protein transport

Table 3.7

Gene ID	Morphology	Gene Name	Gene Description
SPAC1851.02	Class Ib	*slc1*	1-acylglycerol-3-phosphate O-acyltransferase, involved in glycerol
SPAC16E8.08	Class Ib		Sequence orphan
SPAC25G10.03	Class Ib	*zip1*	Transcription factor involved in response to arsenic
SPAC6G9.14	Class Ib		RNA-binding protein
SPBC1604.02c	Class Ib		Sequence orphan
SPCC4F11.03c	Class Ib		Sequence orphan
SPAC20G4.08	Class Ib		Sequence orphan
SPAC1F5.07c	Class Ib	*hem14*	Protoporphyrinogen oxidase
SPAC4H3.14c	Class Ib		Sequence orphan
SPAC22E12.04	Class Ib	*ccs1*	Metallochaperone, involved in cellular copper ion homeostasis
SPBC1685.13	Class Ib	*fhn1*	Involved in plasma membrane organization
SPBC3H7.03c	Class Ib		2-oxoglutarate dehydrogenase (lipoamide)
SPBC15D4.07c	Class Ib	*atg9*	Autophagy associated protein
SPCC777.10c	Class Ib	*ubc12*	Ubiquitin conjugating enzyme
SPBP4H10.19c	Class Ib		Calreticulin/calnexin homolog, involved in intracellular protein transport
SPAC521.05	Class Ib		40S ribosomal protein S8

Table 3.7

Gene ID	Morphology	Gene Name	Gene Description
SPAC4C5.03	Class Ib		CTNS domain protein
SPCC191.05c	Class Ib		nucleoside 2-deoxyribosyltransferase

Table3.7 Class Ib morphological mutant strains identified from version β and version 2 Bioneer *S. pombe* deletion library screen on LNB. All gene descriptions are taken from Gene DB (http://old.genedb.org/genedb/pombe/ last accessed 11/07/2011)

filamentous cells (figure3.5, table 3.8) Strains that were able to form filamentous protrusions but the cells in the filaments were extensively elongated. (IIb) **Strains that had erratically structured filamentous cells** (figure3.6, table 3.9) Strains were able to form filamentous protrusions but the cells in the filaments were no longer uniform in size or shape. Cells varied from bloated and rounded to pear shaped. Not all cells were morphologically aberrant in these filaments.

Four morphologically aberrant classes were identified in this screen. Table 3.10 details the number of strains identified in each morphologically aberrant phenotypic group.

Phenotype	Number of mutants
Class Ia: Unable to form filamentous protrusions	9
Class Ib: Thickened filamentous rope structures	50
Class IIa: Elongated filamentous cells	10
Class IIb: Erratically structured filamentous cells	70

Table 3.10 Number of strains attributed with each morphological defect.

Individual inspection of these phenotypic classes will facilitate further understanding of the invasive process.

3.2.5.i: Class Ia) 9 Strains were unable to form filamentous protrusions

Nine strains were unable to form filamentous protrusions radiating out from the central focus. The morphology exhibited by these strains suggests the invasive process involves a third stage as the strains can adhere (first stage) and invade (second stage), but cannot form complete invasive foci. As these strains were unable to form filamentous protrusions, this newly described tertiary stage will be termed filament formation. Invasive growth of *S. pombe* can therefore be considered a three stage process.

Stage: Primary Secondary Tertiary

Process: Adhesion ⟶ Invasion ⟶ Filament Formation

The mutants within this group are blocked at the tertiary stage of the invasive process. There are nine mutants within this phenotypic group. These exhibit the phenotype with variable severity (table 3.6), which has been designated arbitrary values with +++ being most severe and + being least severe. *Tea2, tea1, tip1, mbo1* encode polarity determining growth factors (Browning et al. 2000; Snaith et al. 2005). *Alg10, kes1* and *Spcc18.10* have not yet been characterised in *S. pombe* so their roles are inferred from homology. Alg10 is a glycosyltransferase that is involved in N- linked glycosylation.

Gene ID	Morphology	Gene Name	Gene Description
SPAPB1A10.14	Class IIa		F-box protein (context dependent), no apparent orthologs
SPBC409.07c	Class IIa	*wis1*	Serine/threonine protein kinase, MAPKK for Sty1 SAPK cascade
SPBC1105.14	Class IIa	*Rsv2*	Involved in transcriptional regulation
SPAC24B11.06c	Class IIa	*sty1*	Serine/threonine protein kinase, Sty1 SAPK cascade, involved in mitotic checkpoint
SPAC6B12.15	Class IIa	*cpc2*	RACK1 homologue Cpc2, involved in protein localisation
SPBC4C3.12	Class IIa	*sep1*	Fork head transcription factor, involved in control of cell separation
SPCC790.03	Class IIa		Rhomboid family protease
SPAC2F7.07c	Class IIa	*rco1*	Histone deacetylase complex subunit, involved in chromatin remodelling
SPCC645.07	Class IIa	*rgf1*	Rho GEF for Rho1, involved in cell wall biogenesis
SPAC1006.09	Class IIa	*win1*	Serine/threonine protein kinase in Sty1 SA MAP kinase signalling cascade

Table 3.8 Class IIa morphological mutant strains identified from version β and version 2 Bioneer *S. pombe* deletion library screen on LNB. All gene descriptions are taken from Gene DB (http://old.genedb.org/genedb/pombe/ last accessed 11/07/2011)

Gene ID	Morphology	Gene Name	Gene Description
SPAC19D5.06c	Class IIb	*din1*	nuclear exonuclease binding protein, involved in rRNA processing
SPAC6B12.05c	Class IIb	*ies2*	Ino80 complex subunit, involved in chromatin remodelling
SPAC4H3.04c	Class IIb		conserved eukaryotic protein
SPBC14C8.08c	Class IIb		involved in translation
SPBC21.02	Class IIb		Conserved hypothetical protein
SPBC16E9.18	Class IIb	*psd1*	phosphatidylserine decarboxylase proenzyme 1, involved in phosphatidylcholine biosynthetic process
SPBC947.03c	Class IIb	*naa38*	NatC N-acetyltransferase non catalytic subunit
SPBC1734.12c	Class IIb	*alg12*	alpha-1,6-mannosyltransferase predicted to be involved in cell wall biosynthesis
SPBC14C8.17c	Class IIb	*spt8*	SAGA-like complex, involved in chromatin remodelling
SPCC1672.06c	Class IIb	*asp1*	inositol hexakisphosphate kinase involved in cortical actin cytoskeleton
SPCC1322.07c	Class IIb	*mug150*	sequence orphan
SPCC126.15c	Class IIb	*sec65*	involved in intracellular protein transport
SPBC36.04	Class IIb	*cys11*	cysteine synthase, involved in cysteine metabolism
SPAC926.03	Class IIb	*rlc1*	myosin regulatory light chain (type II), relieves auto-inhibition of myosin heavy chain function
SPBC27B12.06	Class IIb	*gpi13*	involved in GPI anchor biosynthesis (predicted)
SPAPYUG7.03c	Class IIb	*mid2*	anillin homologue, involved in cell separation
SPAC31A2.11c	Class IIb	*cuf1*	Cu metalloregulatory transcription factor, involved in iron homeostasis
SPAC8E11.02c	Class IIb	*rad24*	involved in DNA damage checkpoint, involved in G2/M transition

Table 3.9

Gene ID	Morphology	Gene Name	Gene Description
SPAC17H9.08	Class IIb		mitochondrial coenzyme A transporter
SPCC794.07	Class IIb		Involved in acetyl-CoA biosynthetic process from pyruvate
SPAC1805.14	Class IIb		sequence orphan
SPAC26F1.10c	Class IIb	*pyp1*	phosphoprotein phosphatase activity, de-phosphorylates Sty1 MAPK
SPAC3G6.01	Class IIb	*hrp3*	involved in chromatin silencing (predicted)
SPBC609.05	Class IIb	*pob3*	FACT complex component (predicted) involved in chromatin silencing
SPAC19G12.08	Class IIb	*scs7*	sphingosine hydroxylase, involved in the hydroxylation of the very long chain fatty acid
SPAC3G9.05	Class IIb		GTPase activating protein, involved in establishment of cell polarity
SPBC1709.11c	Class IIb	*png2*	involved in chromatin silencing and transcriptional regulation (predicted)
SPAC144.02	Class IIb	*iec1*	Ino80 complex subunit, involved in chromatin remodelling
SPCC757.09c	Class IIb	*rnc1*	RNA-binding protein, involved in negative regulation of MAPKKK signal transduction, interacts physically with
SPAC17A2.13c	Class IIb	*rad25*	14-3-3 protein, involved in DNA damage checkpoint, involved in cell wall organization and biogenesis
SPAC222.08c	Class IIb		involved in pyridoxine metabolism (predicted)
SPAC637.06	Class IIb		alpha-1,2-galactosyltransferase, involved in cell wall biogenesis
SPBC2F12.04	Class IIb	*rpl1701*	60S ribosomal protein L17
SPBPJ4664.06	Class IIb	*gpt1*	glycosyl transferase family 8, involved in beta glucan biosynthesis
SPBC1709.18	Class IIb	*tif452*	translation initiation factor, eIF4E (subunit)
SPAC9G1.06c	Class IIb	*cyk3*	involved in cytokinesis (predicted)

Table 3.9

Gene ID	Morphology	Gene Name	Gene Description
SPAC1556.02c	Class IIb	*sdh1*	succinate dehydrogenase (ubiquinone), involved in mitochondrial electron transport
SPBC19C7.02	Class IIb	*ubr1*	ubiquitin-protein ligase (E3)
SPAC24B11.09	Class IIb		conserved eukaryotic protein
SPCC825.01	Class IIb		ribosome biogenesis ATPase
SPBC19G7.10c	Class IIb		topoisomerase associated protein
SPBC21C3.13	Class IIb		40S ribosomal protein S19
SPCC970.10c	Class IIb	*brl2*	ubiquitin-protein ligase E3
SPAC57A10.12c	Class IIb	*ura3*	dihydroorotate dehydrogenase, involved in 'de novo' pyrimidine base biosynthetic process
SPAC688.11	Class IIb	*end4*	Involved in actin cytoskeleton organisation
SPAC5D6.05	Class IIb	*pmc6*	mediator complex subunit, involved in regulation of transcription
SPAC630.14c	Class IIb	*tup12*	transcriptional co-repressor
SPCC777.08c	Class IIb	*bit61*	TORC2 subunit
SPBC365.16	Class IIb		sequence orphan
SPBC365.03c	Class IIb		60S ribosomal protein L21
SPBC83.01	Class IIb	*ucp8*	UBA/EH/EF hand domain protein, involved in actin cortical patch assembly
SPBC1D7.04	Class IIb	*mlo3*	RNA annealing factor, involved in mRNA export from nucleus
SPBC1778.05c	Class IIb		sequence orphan
SPCC550.01c	Class IIb		CHCH domain protein, involved in mitochondrial respiratory chain complex assembly

Table 3.9

Gene ID	Morphology	Gene Name	Gene Description
SPBC1773.01	Class IIb		Involved in cell cycle arrest in response to pheromone (based on homology)
SPBC428.06c	Class IIb	*rxt2*	histone deacetylase complex subunit, involved in chromatin remodelling
SPCC14G10.04	Class IIb		sequence orphan
SPBC16E9.09c	Class IIb		COPII vesicle coat component Erp5/Erp6, involved in ER-Golgi vesicle mediated transport
SPAC1093.01	Class IIb		Conserved protein
SPBPB2B2.14c	Class IIb		*S. pombe* specific DUF999 protein family 8
SPBP35G2.07	Class IIb	*ilv1*	acetolactate synthase catalytic subunit, involved in isoleucin biosynthesis
SPAC3F10.04	Class IIb	*gsa1*	glutathione synthetase large subunit, involved in cell response to stress
SPCC1739.07	Class IIb	*cti1*	substrate-specific nuclear cofactor for exosome activity
SPBC30B4.01c	Class IIb	*wsc1*	transmembrane receptor
SPBC16A3.19	Class IIb	*eaf7*	histone acetyltransferase complex subunit, involved in chromatin remodelling
SPBC19G7.02	Class IIb		4-amino-4-deoxychorismate lyase, involved in L-phenylalanine metabolic process
SPAC3G9.08	Class IIb	*png1*	Involved in DNA repair
SPAC2E1P5.02c	Class IIb	*mug109*	Conserved hypothetical protein
SPAPB17E12.05	Class IIb	*rpl3703*	60S ribosomal protein L37
SPAC637.10c	Class IIb	*rpn10*	19S proteasome regulatory subunit Rpn10

Table3.9 Class IIb morphological mutant strains identified from version β and version 2 Bioneer *S. pombe* deletion library screen on LNB. All gene descriptions are taken from Gene DB (http://old.genedb.org/genedb/pombe/ last accessed 11/07/2011)

Kes1 encodes an oxysterol binding protein that is involved in ergosterol biosynthesis. Spcc18.10 is proposed to be a pyridoxine-pyridoxal-pyridoxamine kinase (http://old.genedb.org/genedb/pombe/). *Asl1* encodes a putative glucosyl hydrolase (de Groot et al. 2007). Finally, *fkh2* encodes a fork head transcription factor that up regulates gene expression in the M-G1 phase of the cell cycle (Bulmer et al. 2004).

3.2.5.ii: Class Ib) 50 strains had filamentous protrusions with thickened rope structures

50 strains appeared to have more cells forming the filamentous ropes behind the leading filament. This gave a thickened rope appearance (figure 3.5, table 3.7). 36 of these strains represent proteins have either general housekeeping roles, are conserved hypothetical proteins or sequence orphans (table 3.7). Five strains have transcriptional or nuclear roles. The remaining nine strains represent proteins that may have interesting roles during invasion and will be discussed further. Three of these proteins (Pac2, Spac1093.03, Hse1) are involved in sexual development triggered by low nitrogen (Kunitomo et al. 1995; Onishi et al. 2007). Mal3 is involved in maintaining microtubule integrity (Busch et al. 2004). Tea4 is a cell polarity factor required for bipolar growth (Tatebe et al. 2005). Pek1 functions in the cell integrity mitogen activated protein kinase (MAPK) pathway (Sugiura et al. 1999). Spn3 is an experimentally characterised septin (An et al. 2004). Acp1 is an F-actin capping protein that serves to prevent association or dissociation of globular actin monomers (G-actin) to the barbed ends of filamentous actin (F-actin) (Nakano et al. 2006). Fhn1 is involved in the organisation of the plasma membrane in areas that are symporter rich (http://old.genedb.org/genedb/pombe/).

3.2.5iii: Class IIa) 10 strains had filamentous cells that were elongated

Ten strains had elongated filamentous cells (figure 3.5, table 3.8). Three of these strains represent components of the stress-activated mitogen activated protein kinase (MAPK) cascade, Wis1-Win1-Sty1 (Samejima et al. 1997). The morphology of *sep1Δ* differs to that of the other class IIa stains (figure 3.5). Sep1 is a fork head transcription factor that controls cytokinesis (Ribar et al. 1999). Rco1 is part of the histone deacetylase complex Rpd3 (http://old.genedb.org/genedb/pombe/). Spapb1A10.14 is an F-box protein, that has no orthologues therefore its role is inferred from homology. SPCC790.03 is a conserved hypothetical rhomboid protease (http://old.genedb.org/genedb/pombe/). Cpc2

elicits a morphological response of the protein kinase C pathway via alteration of mRNA translation (Nunez et al. 2010). Rgf1 is a GEF for Rho1 (Garcia et al. 2009).

3.2.5iv: Class IIb) 70 strains had filamentous cells with erratic structures

70 strains were able to form filamentous protrusions but the cells within them were not uniform in shape and size. Some cells in these filaments were erratic in shape and size (figure 3.6, table 3.9). 49 strains within this group represent hypothetically conserved, sequence orphans, or general housekeeping genes. 21 of the mutant strains represent proteins of potential interest in the invasive response.

Seven of these have roles in cytokinesis, ranging from regulators, such as Psd1, which it thought to control spatial organization of cytokinesis (Luo et al. 2009), to effectors such as Rlc1 which is a myosin regulatory light chain (Sladewski et al. 2009). Mid2 functions in the assembly of the actomyosin contractile ring (Petit et al. 2005) and Asp1 is involved in cortical actin cytoskeleton organization (Feoktistova et al. 1999). Spt8, Sep11 and Ace2 are thought to control cytokinesis at a transcriptional level (Alonso-Nunez et al. 2005; Batta et al. 2009). Rad24 and Rad25 are DNA checkpoint proteins that have been shown to be involved in regulation of cytokinesis (Ishiguro et al. 2001; Mishra et al. 2005). Cyk3 is a protein involved in cytokinesis, though it has not been experimentally characterised so as yet the exact role is unclear (http://old.genedb.org/genedb/pombe/). Mak3 is a histidine kinase (Aoyama et al. 2001). Cuf1 is a transcription factor responsible for the transcription of copper uptake genes in response to reduced copper levels (Rustici et al. 2007). Pyp1 is a protein phosphatase for the Sty1 MAPK response (Dal Santo et al. 1996). SPAC3G9.05 has not been experimentally characterised in *S. pombe* but is proposed to be the orthologue of *S. cerevisiae* SPA2 which functions in actin cytoskeleton organisation during polarised growth (Sheu et al. 1998). Rnc1 is an RNA binding protein that is involved in negative regulation of the cell-integrity response MAPK (Sugiura et al. 2003). SPAC222.08c is a predicted glutamine amino-transferase subunit and is involved in pyridoxine (PN) biosynthesis and SPAC637.06 is proposed to be a α1, 2 galactosyltransferase which is involved in cell wall biogenesis (http://old.genedb.org/genedb/pombe/). End4 is involved in cellular growth zone determination (Castagnetti et al. 2005). Bit61 is part of the TOR signalling cascade (Hayashi et al. 2007), Ucp8 is involved in actin cortical patch assembly (http://old.genedb.org/genedb/pombe/) and Gsa1 is involved in

glutathione biosynthesis (Wang et al. 1997). Finally, Wsc1 is a transmembrane receptor involved in organization of the actin cytoskeleton in response to stress (Philip et al. 2001).

The aberrant morphology of these strains could arise from single cell growth defects. To address this question five strains that exhibit erratically structured filamentous cells were analysed for morphological aberrations as single cells (figure 3.6). Four of these strains (figure 3.6 Bi,Di,Ei,Fi) exhibited aberrant morphologies, whereas one strain (figure 3.6 Ci) exhibited a morphology indistinguishable from *S. pombe* 972. This suggests that class IIb is a heterologous mix of strains, some of which exhibit aberrant morphologies specifically during invasive growth and some whose defects also occur during single cell growth.

The identification of these 70 strains that have erratically structured filamentous cells concludes the description of aberrant invasive morphologies. During this screen it became apparent that strains were capable of exhibiting aberrations in invasive efficiency as well as morphology.

3.2.5v: Five strains exhibited multiple phenotypes

177 strains were classified with aberrations in invasive efficiency or morphology. Five of these strains exhibited aberrations in both. Defects in invasive efficiency represent problems during the secondary stage of invasion, whereas morphological aberrations represent problems in the tertiary stage. Therefore strains defective in both represent the processes which are important for both stages of invasion. Four strains (*atg9Δ, spac6g9.14Δ, adn1Δ, pyp1Δ*) exhibited poor invasive efficiency and one was hyper-invasive (*wsc1Δ*). Wsc1 is a transmembrane receptor that activates the stress activated MAPK signalling cascade (Philip et al. 2001). Atg9 is an autophagy associated protein involved in membrane trafficking, SPAC6G9.14 has not yet been characterised in *S. pombe* but is orthologous to *S. cerevisiae* PUF4 which is an RNA binding protein (http://old.genedb.org/genedb/pombe/). Adn1 is a transcription factor involved in cellular adhesion (Dodgson et al. 2009). Finally Pyp1 is a phosphatase for Sty1 which is part of the stress-activated (SA) MAPK cascade (Dal Santo et al. 1996).

The screen has classified 177 strains with aberrations in efficiency or morphology of the invasive process. The large number of strains utilised throughout this study provides potential for strain contamination, therefore it is important to validate the origin of the phenotypes displayed.

3.2.6: Spbc1289.15 is not required for the invasive growth in *S. pombe*

Linder *et al.* (2008) suggested that Spbc1289.15, a predicted cell surface glycoprotein, is involved in invasive growth (Linder et al. 2008). They based this prediction on its homology to *S. cerevisiae* FLO11 which is an essential adhesin in the invasive response of *S. cerevisiae*. *Spbc1289.15Δ* is not in either of the Bioneer deletion libraries but it was purchased as a single diploid deletion strain from Bioneer. This diploid deletion strain has a meiotic defect which results in its stability as a diploid. Transformation of the diploid strain with pON177 rescues the meiotic integrity of this strain (Kim et al. 2010). The strain was then induced to sporulate and haploid deletion strains were recovered. This allowed investigation into the role of Spbc1289.15 in invasive growth. *Spbc1289.15Δ* exhibited strong invasive growth (data not shown) implying SPBC1289.15 is not an essential component of invasive growth, though it may be a redundant protein in the process.

3.2.7: Confirmation of phenotype origin

This screen utilised thousands of strains each with a single gene deletion that was selectable by G418 resistance. The specific gene deleted in each strain is outlined in a reference spreadsheet. The high number of strains used in this study provides the possibility for strain contamination. To ensure the strains attributed with aberrant phenotypes represented their described genes in the reference spreadsheet, two forms of confirmation were used; PCR and backcrossing. 18 strains were subjected to PCR analysis, and an additional ten strains were backcrossed to *S. pombe* 972.

3.2.7i: Backcrossing

The aim of this procedure is to ensure the specific gene deletion, selectable by G418 resistance, causes the described phenotype. This is done by checking the phenotype segregates with G418 resistance after mating and sporulation. *S. pombe* has a high rate of homologous recombination, so if two phenotypes (G418 resistance and mutant phenotype) are not caused by the same gene then when the strain undergoes mating and subsequent sporulation, these phenotypes will segregate away in the progeny.
Ten strains were randomly selected for backcrossing. These were either analysed by tetrad dissection or random spore analysis (Materials and Methods). G418 resistance and mutant phenotype co-segregation was seen in all ten strains tested (data not shown).

As a random sample of ten all segregated with G418 resistance the phenotypes of the remaining strains are likely to be attributable to the gene deletion specified in the spreadsheet.

3.2.7ii: PCR

The aim of this procedure was to ensure the deleted gene of a specific strain correlated to the described gene in the reference spreadsheet. The strains were created by deletion of the specific gene open reading frame (ORF) via insertion of KanMX4. Genomic DNA was extracted and used in PCR with the corresponding strain specific primer. Primers (see material and methods) were designed roughly 200bp up stream of the ORF start site and the reverse primer was intrinsic to KanMX4. Therefore, a PCR fragment would only be amplified if the DNA template corresponded to the strain specific primer. 18 strains were subject to PCR analysis and each produced a product of correct size. Nine of these amplification products are shown in figure 3.7. The size of fragments amplified during PCR vary slightly, due to the variable insertion position of KanMX4, as sometimes it is not inserted directly at the start of the ORF (http://pombe.bioneer.co.kr/technic_infomation/construction.jsp). This accounts for the slight variability for fragment size in lane 1,2, 3 and 4 (figure 3.7). As all strains tested had their specified gene deleted the remaining library strains are likely to be correct.

3.3: Discussion

3082 *S. pombe* deletion library strains were screened for aberrations in the invasive process. This resulted in the identification of 177 strains with aberrations in invasive efficiency, morphology or both. Furthermore, it facilitated the description of a third stage of the invasive process, filament formation. Prior to the analysis of the strains identified in the screen, the methodology must be considered. The large scale screening process required the preparation of media that supported reliable invasion as well as strains capable of invasion on LNB.

3.3.1: Preparations for the screen

3.3.1i: Media preparation

The agar constituent of media can drastically affect invasive efficiency. Why is this? Agar is a polysaccharide of agarose and agropectin, which can be methylated, sulphated

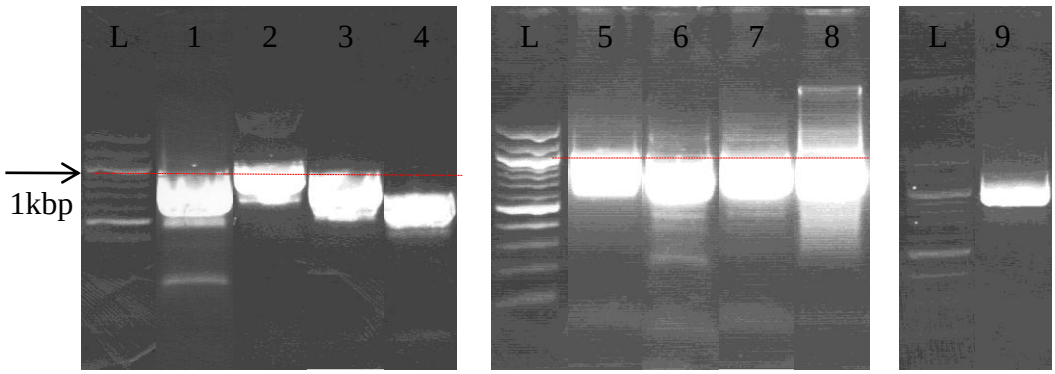


Figure 3.7 PCR confirmation of a random selection of mutants from both version β and 2 of *S. pombe* Bioneer deletion library. Fragment size should be 988bp.L) Ladders,100bp 1) *SPAC3H5.11Δ* 2) *SPBC18H10.19Δ* 3)*rad25Δ* 4) *SPBC3B8.08Δ* 5) *SPBC1539.06Δ* 6) *SPCC1322.07cΔ* 7) *ace2Δ* 8) *sty1Δ* 9) *rad24Δ*. The red dotted line indicates the expected fragment size. The fragment size may vary slightly as the exact insertion point of the KanMX4 cassette is not known.

and pyruvylated and these modifications affect the structure and rigidity of the agar (http://www.fao.org/). Agar is extracted from natural substances so there is limited control on its composition and so varying sources will have varying rigidity. It seems weak agar rigidity is required for invasion, thus decreasing the agar concentration of a more rigid source yields better invasive efficiency.

Invasion is strongest on media that is left to set whilst covered up. The wetness of the media would be affected by variation of the setting conditions. The covered conditions increase the required setting time and this could affect the structure of the media. The covering effect could also be due to the decrease in water loss from the media thus altering its rigidity.

The optimization of media preparation facilitated the screening of the Bioneer *S. pombe* deletion libraries for aberrations in the invasive process.

3.3.1ii: Coverage of the screens

3082 strains, each with a single gene deleted, were analysed for aberrations in the invasive process. 177 strains were classified as phenotypically aberrant in one or more of seven categories. This presumably represents a large proportion of the genes involved in the invasive process, though not all of them as it is highly probably there is much functional redundancy. Additionally, the Bioneer deletion libraries do not cover the entire *S. pombe* genome.

There are 4876 protein coding sequences (CDS) in the *S. pombe* genome. 1274 of these encode essential proteins (http://old.genedb.org,), therefore deleted strains are not available. Over-expression experiments could facilitate analysis of the functional role of the essential genes.

This study analyses 86% of the non-essential genes. This leaves 14% that have not been analysed, some of which can be accounted for by the conversion to prototrophy (see section 3.2.2). These 210 strains account for 5%, leaving 9% or 310 genes whose role in invasive morphology has not been analysed.

Therefore 91% of the Bioneer *S. pombe* deletion library that are capable of prototrophic conversion have been analysed for aberrations in the invasive process. The identification of 177 mutant strains and description of four novel phenotypes means significant progress in understanding the invasive process has been made.

The Bioneer *S. pombe* version β and version 2 deletion libraries are large collections of strains therefore their use requires rigorous attention to detail to avoid strain contamination. Quality control techniques ensure the data produced is reliable.

3.3.2: Quality control of the screen

3.3.2i: Diploid instability avoids false negative result

The conversion of the Bioneer *S. pombe* deletion libraries to prototrophy involved high throughput mating, which involved the generation of diploids. The screen relies on these diploids going through meiosis and forming haploid spores. There is no selective pressure against the survival of parental diploids and analysis of a recessive mutant diploid strain could produce a false negative result. However, as no colony purification step is involved this would require the majority of cells to be diploid. A diploid screening stage could be employed but this has been addressed in a similar screen in this laboratory, with the conclusion that no diploids survived, therefore this screening stage was considered redundant.

Diploid instability assures the elimination of a false negative result and therefore the phenotype exhibited by a specific strain, but can all the gene deletions that cause these phenotypes be trusted?

3.3.2ii: Discrepancies between the version β and version 2 of the Bioneer *S. pombe* deletion libraries

The screen utilised version β and version 2 of the Bioneer *S. pombe* deletion libraries. The version 2 library provided additional strains for invasive, morphological analysis. Version 2 deletion library contained the majority, but not all of the strains in version β. There are 298 strains in version β that are not in version 2. The absence of these strains from version 2 suggests their deletion may have been incomplete in version β as further strain confirmation was carried out in the creation of the version 2 library. For this reason, the 4 strains that were attributed with a phenotype from the version β screen, that were not in version 2 have since been discarded from the data. These were all conserved hypothetical proteins.

The PCR and back crossing results presented in section 3.2.6 confirms origin of phenotype and assures the reliability of the screen results. This reliability now allows correlation analysis between phenotype predictions and the screen result.

3.3.2iii: *S. pombe* based phenotype predictions

Previous research into invasive growth of *S. pombe* has implicated pathways and specific proteins in invasion so it is possible to predict the phenotypic outcome of specific strains in the screen. The correlation of these predictions with the actual screen results can serve as a form of quality control.

The original description of *S. pombe* invasive growth implicated the glucose activated cAMP/protein kinase A (PKA) signalling cascade in the process (Amoah-Buahin et al. 2005) therefore representative strains of essential components are predicted to be non-invasive.

Git3, Gpa2 and Git5, the G protein coupled receptor, G protein α subunit and G protein β subunit respectively, function to increase intracellular cAMP and activate PKA (Hoffman et al. 1991). A non-invasive or poorly invasive phenotype is presented by these three representative deletion strains in the screen (table 3.3, 3.4). In contrast *pka1Δ, git11Δ* which represent the PKA catalytic subunit and the G protein γ subunit respectively (Hoffman et al. 1991; Landry et al. 2001) were able to form invasive structures. The *git11Δ* and *pka1Δ* results do not corroborate with the work of Amaoh-Buahin et al, 2005, who originally described the involvement of the cAMP signalling cascade in invasive growth. This may suggest the genes have not been completely deleted in these strains. Alternatively the variation in phenotype may arise from the slight variation in the method of media preparation.

As the representative strains of the receptor (*git3*Δ) and early transducers (*gpa2Δ, git5Δ*) of the cAMP signalling cascade exhibit non-invasive phenotypes but the subsequent transducer (*pka1Δ*) does not this may suggest an alternative effector of the cAMP signal is involved in invasive growth. However, as Pka1 is the only characterised downstream effector of cAMP this seems unlikely.

Fep1Δ has previously been described as non-invasive (Prevorovsky et al. 2009). The *fep1Δ* mutant exhibited a non-invasive phenotype in the screen.

amt1Δ, amt2Δ and *amt3Δ* (Mitsuzawa 2006) have been described as non-invasive, however these three strains were all capable of invasion in the screen. This difference can be attributed to the difference in density of cells used for invasive growth. Mitsuzawa *et al*, 2006, utilised a low density of cells, whereas the screen utilized high densities. The John Armstrong laboratory has previously shown that difference in cell density can affect invasive efficiency (un-published data).

A previous study of the version β Bioneer *S. pombe* deletion library identified 12 strains as non-invasive (Dodgson et al. 2009). Five of these strains also exhibit a non-invasive phenotype in this screen. As the two screens used different conditions the results would be expected to share some common factors, though not be identical.

Five strains (*tea1Δ, tea2Δ, tip1Δ, mal3Δ, tea4Δ*) have been previously described as morphologically aberrant (Dodgson et al. 2009) and these were also characterised as morphologically aberrant in this screen.

The phenotypic predictions of 25 strains are correct for 14 and incorrect for 11. However, of the 11 that are incorrect nine can be accounted for by differences in invasive conditions used. Therefore, the correlations of predicted phenotype with screen result strengthen the reliability of the screen.

The reliability of the screen permits analysis of the resultant phenotypic groups. The first group to be analysed are those defective in the primary stage of invasion, adhesion. This screen has identified many genes that cannot be attributed with an immediately obvious role in the invasive process. They shall not be considered further, though this does not detract from their importance in the invasive process. Therefore, only genes that present potential insights into the invasive process will be discussed.

3.3.3: Strains that exhibit aberrations in the primary and secondary stages of the invasive process

3.3.3i: Non-adhesive strains

Adhesion is the primary stage in the invasive process (Dodgson et al. 2009). In this stage cells physically stick to the agar, which may be facilitated by the galactose component of the agar polymer. Presumably, proteins protruding out of the *S. pombe* cell wall attach to the galactose residues.

Six strains (*sol1Δ, dep1Δ, snf5Δ*, *adn2 Δ, adn3 Δ* and *hsr1Δ*) that exhibit an adhesion defective phenotype on LNB have nuclear based roles (table 3.2). Sol1, Dep1 and Snf5 are all involved in chromatin remodelling (Monahan et al. 2008) and Adn2, Adn3 and Hsr1 are transcription factors (http://old.genedb.org/genedb/pombe/). This suggests that adhesion is regulated by transcriptional changes. Additionally, *pyp1Δ* is also adhesion defective. Pyp1 is an inhibitory protein phosphatase for Sty1 (Shiozaki et al. 1995), a stress-activated MAPK that generally functions via translocation to the

nucleus resulting in gene expression alteration (Reiter et al. 2008). This implicates Sty1 as a negative regulator of the adhesive response on LNB.

Fta5Δ and *atg14Δ* are also unable to adhere on LNB. Atg14 is a phosphatidylinositol-3 kinase (PI-3-K) that is responsible for the production of phosphoinositide-3-phosphate (PI-3-P), which then signals to downstream effectors resulting in an autophagic response (Obara et al. 2006). Autophagy is the degradation of cytoplasmic contents for use as energy under nutrient deprived conditions (Mukaiyama et al. 2010). Atg14 is a member of PI-3K complex 1, which is composed of two additional subunits, Vps34 and Vps30 (Obara et al. 2006); *Vps34Δ* did not exhibit an aberrant invasive phenotype and *vps30Δ* was not in the library, therefore was not tested. The lack of adhesion defective phenotype of *vps34Δ* suggests that Atg14 functions in an autophagy independent manner during adhesion. Fta5 is a cell surface glycoprotein required for cell-cell adhesion.

The analysis of the non-adhesive strains proposes a mechanism where transcriptional control and Atg14 production of PI-3-P results in Fta5 expression on the cell surface. This expression of Fta5 may be required for invasion specific adhesion.

par1Δ is the only strain that exhibits hyper-adhesive attachment. *par1* encodes the regulatory subunit for protein phosphatase 2A (PP2A) and is a negative regulator of the septation initiation network (SIN) (Jiang et al. 2000). Potentially PP2A targets a protein that affects adhesive regulation. Atg14 (a kinase) and PP2A (a phosphatase) could be acting antagonistically on an as yet uncharacterised protein that is required for adhesion.

This concludes the analysis of the strains exhibiting a defect in the primary stage of the invasive process, adhesion. The following stage of the invasive process is invasion.

3.3.3ii: Non-invasive strains

The 19 genes represented by the non-invasive strains were subjected to GO over-representation analysis by BiNGO plug-in for Cytoscape (figure 3.4a, appendix 3.1). The presence of "cell adhesion" GO term on the BiNGO diagram is due to *fta5* and *ogm1* (appendix 3.1) suggesting the encoded proteins are involved with cell-agar adherence as cell adhesion is the preliminary stage of invasion. *ogm1Δ* did not exhibit an adhesion defective phenotype. This suggests its encoded protein is not as important as Fta5 in this process. Eight of the 17 strains attributed with a non-invasive phenotype had nuclear based roles (table 3.3) and account for the 2 nuclear based GO terms on the

BiNGO diagram (figure 3.4a, appendix 3.1). This suggests transcriptional regulation is the main method of induction of invasion.

In addition to BiNGO analysis, specific analysis of individual strains attributed with the non-invasive phenotype may be useful in understanding this secondary stage of the invasive process.

3.3.3iia: Fta5 may be an adhesin required for invasion

Fta5 was proposed to be a kinetochore core protein that interacts with Sim4 and Mal2 (Sim four and Mal two associated protein 5) (Liu et al. 2005). However, the evidence for the proposed kinetochore function was weak and Fta5 has been more recently described as a cell surface glycoprotein that has a GLEYA domain (Linder et al. 2008). GLEYA domains are lectin-binding like domains that are located in the C terminus of adhesins (Linder et al. 2008). The GLEYA name is derived from the conserved residues exhibited by seven of the putative adhesins in *S. pombe*. The GLEYA domain is related to the lectin-like binding domain of adhesins in *S. cerevisiae* which is part of the wider PA14 carbohydrate binding domain (Linder et al. 2008). Based on these similarities and the inability of Fta5 to adhere or invade during invasive growth, Fta5 is probably an adhesin required for invasion.

3.3.3iib: Remodelling of the cell wall and cell membrane is required for invasion

Ogm1 encodes a cell wall modification protein (Willer et al. 2005) that could be responsible for Fta5 presence on the cell surface. Invasive signal transduction could result in cell wall remodelling, which facilitates invasive growth. Ergosterol is a major cell membrane constituent and Ccr1 transfers electrons to various enzymes within the ergosterol biosynthetic process, therefore the non-invasive phenotype exhibited by *ccr1Δ* may be attributed to defects in cell membrane synthesis.

3.3.3iic: The Ras1 signalling pathway may be required for invasion

Invasion is probably a result of a number of cellular processes and remodelling the cell wall (described above) may be one of these. The regulation of the actin cytoskeleton is another process which probably contributes to invasive growth. Ccr1 is a NADH –cytochrome p450 reductase (http://old.genedb.org/genedb/). The *S. cerevisiae* orthologue, NCP1, is required for sterol synthesis and functions in polarised growth through interactions with *Sc*Cdc42 (Lin et al. 2009). *S. pombe* Cdc42 is activated by

Ras1 and relieves auto-inhibition of formin For3 (Martin et al. 2007). For3 is required for efficient invasive growth as *for3Δ* exhibits a poorly-invasive phenotype (table 3.4) (Dodgson et al. 2009). Perhaps the sterol synthesis regulated by Ccr1 and actin cable polarization mediated by For3 are inter-reliant during invasive growth. Therefore, if a similar interaction of *Sp.*Ccr1-*Sp.*Cdc42 takes place as seen in *S. cerevisiae* between *Sc.*NCP1-*Sc.*CDC42, then the *ccr1Δ* phenotype may arise through an inability to activate For3 and reduced sterol synthesis. Additionally, Efc25 is a putative GEF for Ras1 which is responsible for *Sp.*Cdc42 activation (Tratner et al. 1997). This further implicates Ras1 signalling in control of invasive growth. The phenotype displayed by these strains suggest For3 activation is essential for invasion. However, For3 is also activated in single cell growth, therefore there must be a change orientated with For3 to cause invasion. *Ras1Δ* was not tested for aberrations in invasion as it was not recovered from the conversion to prototrophy (section 3.2.2, appendix 3.3).

3.3.3iid: Asymmetrical protein localisation may be involved in invasion

Spac4g8.03c is a RNA binding protein that is capable of localising mRNA to specific cellular sites, which has been shown to be crucial for asymmetrical cellular development (Elson et al. 2009). Cellular asymmetry is important for invasive growth (Dodgson et al. 2010) so Spac4g8.03c may potentially function in coordination of cellular asymmetry thus facilitating the invasive response.

3.3.3iie: The cAMP signalling cascade is required for invasion

Git3 is the membrane bound G protein coupled receptor of the glucose activated cAMP signalling cascade (Welton et al. 2000). This cascade has previously been shown to be crucial in low nitrogen induced invasion (Amoah-Buahin et al. 2005). LNB invasive media has a high glucose level, therefore this pathway is active during invasion. The cAMP signal may combine with an additional low nitrogen induced signal to result in invasion.

The analysis of non-invasive strains allows a number of conclusions to be drawn about the secondary stage of the invasive process:

1) Fta5 is a potential cell surface adhesin involved in invasion.

2) Transcriptional regulation appears to be the main form of invasive induction.

3) Signal transduction most probably results in modification of the cell wall to facilitate invasion.

4) Ras1 signalling may be required for invasion.

Additionally to these newly drawn conclusions this data re-confirms the involvement of the glucose activated cAMP signalling cascade in invasion.

Strains attributed with a non-invasive phenotype are blocked at the secondary stage of the invasive process. This block is less severe in strains that are poorly invasive.

3.3.3iii: Poorly invasive strains

15 strains exhibited a poorly invasive phenotype. These strains represent cellular processes that, although not essential for invasive growth, are involved.

The genes represented by the 15 poorly-invasive strains were subjected to BiNGO analysis (figure 3.4b). As with the BiNGO diagram of the non-invasive strains, transcriptional control is represented, strengthening the conclusion that transcriptional control is the predominant form of invasive regulation.

For3 is formin that is responsible for actin cable assembly and localises to sites of cellular growth (Feierbach et al. 2001). *For3Δ* was identified in a previous screen for strains aberrant in invasion (Dodgson et al. 2009). The inability of *for3Δ* to invade on LNB media suggests it is essential for invasion. Actin cables serve as the delivery tracts for vesicles that fuse with the plasma membrane and facilitate growth (Pruyne et al. 2000).

The visual analysis that identified the non-invasive and poorly-invasive phenotypic groups also identified strains that exhibited a hyper-invasive phenotype.

3.3.3iv: Hyper-invasive strains

Nine strains exhibited a hyper-invasive phenotype (table 3.5, figure 3.2). These strains represent potential negative regulators of the invasive process.

3.3.3iva: Calcineurin is a negative regulator of invasion

Cnb1Δ is proposed to be the regulatory subunit of calcineurin (http://old.genedb.org/genedb). Calcineurin is a calcium/calmodulin dependent protein phosphatase involved in cellular morphogenesis (Yoshida et al. 1994). The regulatory subunit is responsible for binding calcium and activating the catalytic subunit. Therefore, in the *cnb1Δ* strain, calcineurin signalling is turned off. This suggests calcineurin is a negative regulator of invasion. Calcineurin can transduce signals

through Prz1-dependent and independent pathways (Hirayama et al. 2003). *Prz1Δ* did not exhibit an aberrant phenotype in the screen. This implies inhibition of invasion by calcineurin is transduced via the Prz1-independent pathway.

3.3.3ivb: Regulation of cell wall biosynthesis is important in invasion

There are two hyper-invasive strains that represent proteins involved in cell wall biosynthesis, *spbc1198.06cΔ* and *wsc1Δ*. A change in the cell wall biosynthetic process could alter the balance of cell wall constituents resulting in prevalence of a constituent that is preferable for invasive growth.

3.3.3ivc: Rho1 signalling is a negative regulator of invasion

Wsc1 is proposed to be a transmembrane receptor involved in actin cytoskeletal organization (http://old.genedb.org/genedb/pombe/). In *S. cerevisiae* WSC1 acts upstream of *Sc*RHO1 the homologue of *Sp.*Rho1 (Philip et al. 2001). *Sp.*Rho1 can regulate multiple separate cellular events; cell-integrity MAPK signalling (Garcia et al. 2009), β glucan synthase activity (Arellano et al. 1996) and Pck2 signalling (Arellano et al. 1999). 1,3 β glucan synthase is the major producer of 1-3 β glucan which is the major constituent of fungal cell walls (Philip et al. 2001). Without *Sc*WSC1 1,3 β glucan production decreases. If the same is true in *S. pombe* a decrease in 1,3 β glucan in the cell wall appears to favour invasion. Alternatively, the *wsc1Δ* phenotype could arise from one of the other pathways regulated by Rho1. *Sc.*WSC1 activates *Sc.*RHO1 (Philip et al. 2001), therefore *Sp.wsc1Δ* phenotype suggests one these pathway negatively regulates invasion. The cell-integrity MAPK is composed of three proteins (Mkh1, Pek1 and Spm1) that are sequentially activated by phosphorylation (Zaitsevskaya-Carter et al. 1997). None of these deletion strains presented an efficiency defective phenotype in the screen, therefore it seems unlikely Rho1 mediates the signal through this pathway. Rho1 has also been shown to activate Pck2 which transduces the signal to Cpc2 (Arellano et al. 1999; Won et al. 2001). *Cpc2Δ* exhibited a mutant filament morphology (section 3.3.4iiib) which correlates with negative regulation of growth, therefore it seems likely Rho1 negatively regulates invasion via Pck2 and Cpc2 signalling.

Wsc1 is a cell wall integrity sensor that is proposed to probe the extracellular matrix (Rajavel et al. 1999). Potentially, this matrix probing may determine which surface-cells

invade. Invasion normally predominates at the periphery of a colony so perhaps Wsc1 is responsible for activating a signalling pathway that results in inhibition of invasion for those cells that are centrally located within the colony. The central location of these cells means they would not be able to forage for nutrients as well as the peripheral cells.

Rga8 is a Rho1 GTPase activating protein (GAP) (Yang et al. 2003). Normally, GAPs function via activation of the intrinsic GTPase which inactivates their targets. This would result in a constitutively "on" signal in *rga8Δ*. However Rga8 has been shown to have a positive functional interaction with Rho1 (Yang et al. 2003). This means that the *rga8Δ* phenotype correlates with the *wsc1Δ* phenotype and that Rho1 signalling negatively regulates invasion.

3.3.3ivd: Stimulation of invasion may co-incide with inhibition of mating

Rcd1 is required for sexual development. *S.pombe* can be induced to mate by either low carbon or low nitrogen, Rcd1 is required specifically in response to low nitrogen (Okazaki et al. 1998). Under low nitrogen conditions Rcd1 induces Ste11, which is the main transcription factor in control of sexual development in *S. pombe*. Low nitrogen induces invasive growth as well as mating and the differentiation between these two separate responses is not properly understood. Potentially, as well as inducing Ste11 for the mating response, Rcd1 could also repress constituents of the invasive response, and so in *rcd1Δ* mutants this repression is not taking place resulting in hyper-invasion.

3.3.3ive: Regulation of cytokinesis is required for invasion

The only strain representing a transcription factor that exhibited a hyper-invasive phenotype was *ace2Δ.* This is in contrast to the strains exhibiting a non-invasive phenotype, where transcriptional activity was strongly represented. This suggests that transcriptional control is involved in the induction of the invasive response, but not as strongly in the regulation of it. Ace2 is responsible for up-regulating the expression of genes involved in cell separation (Alonso-Nunez et al. 2005), suggesting loss of cell separation results in stronger invasion. Alternatively, Ace2 could be responsible for the transcription of a specific gene whose protein product negatively regulates invasion. In this scenario when *ace2* is deleted this negative regulator of the invasive growth response is no longer up-regulated resulting in hyper-invasion.

Analysis of the hyper-invasive strains allows some conclusions to be drawn about the regulation of invasion:

1) Cell wall composition is important during the invasive process.
2) Calcineurin negatively regulates invasion via a Prz1 independent pathway.
3) Rho1 mediated signalling negatively regulates invasive growth.
4) Regulation of invasion may be largely by post-transcriptional rather than by transcriptional control.

3.3.3v: Conclusions drawn from the analysis of strains that were aberrant in invasive efficiency

The analysis of the hyper-invasive strains has implicated the Rho1 signalling pathway as a negative regulator of invasion which may use the integrity of the cell wall to activate signalling pathways that determine which cells invade. Additionally, calcineurin has been implicated as a negative regulator of invasion. Analysis of the non-invasive and poorly-invasive strains has strengthened the evidence for the requirement of cAMP signalling for invasion. Additionally it has suggested Ras1 signalling is required for invasion. These regulatory pathways probably combine to achieve the invasive growth response.

This concludes the analysis of strains defective in the primary and secondary stage of the invasive process. Microscopic classification of the aberrant strains allowed the description of a tertiary stage to the invasive process, filament formation. Strains presenting novel morphological aberrations within the tertiary stage of the invasive process will now be considered.

3.3.4: Strains that exhibit aberrations in the tertiary stage of the invasive process

138 strains were identified from the screen that exhibited one of four morphologically aberrant phenotypes. The identification of Class Ia strains that were unable to form filamentous protrusions allows the description of a novel third stage in the invasive process.

Stage:	Primary		Secondary		Tertiary
Process:	Adhesion	⟶	Invasion	⟶	Filament Formation

44 strains exhibited aberrations in the secondary stage of invasion in comparison to 138 strains that exhibited aberrations in the tertiary stage. The higher number of strains classified with aberrations in the tertiary stage of the invasive process implies that this stage is more complex. Strains exhibited aberrations in the combined morphology of the filaments (Class I) as well as the individual cell morphology (Class II). The representation of nuclear proteins was stronger in the secondary stage strains than the tertiary, which suggests transcriptional control does not predominate during the tertiary stage.

Analysis of the novel phenotypes described by the screen will aid the characterization of the tertiary stage. The first to be considered are those that are unable to form filamentous protrusions.

3.3.4i: Class Ia) Strains that were unable to form filamentous protrusions

Nine strains were able to invade the media but were unable to form filamentous protrusions. These are blocked at the tertiary stage of invasive growth, filament formation. Analysis of the individual strains exhibiting this phenotype may highlight the processes involved.

3.3.4ia: Growth polarity determinants are essential for the tertiary stage of invasive growth

Four strains, *tea1Δ, tip1Δ, tea2 Δ* and *mbo1Δ*, represent microtubule-associated growth polarity determining proteins. Three of these (*tea1Δ, tip1Δ, tea2 Δ*) had previously been described as morphologically aberrant (Dodgson et al. 2009). Filament formation involves a unique monopolar growth pattern (Dodgson et al. 2010) and Tea2, Tea1, Tip1 and Mbo1 play an essential role in this process. Tea2 is a kinesin microtubule motor that is central to intracellular transport. Tea 2 "walks" along the microtubule carrying cargo proteins for delivery to the cell tip (Browning et al. 2000). This delivery appears to be more important for the tertiary stage of the invasive process than for the secondary stage (figure 3.8). Tea1 is required for the restriction of microtubule growth at the cell tip – without Tea1 the microtubules curve as they reach the pole of the cell instead of starting to depolymerise (Behrens et al. 2002). Potentially this may result in the filaments bending back on themselves and therefore not being able to form filamentous protrusions (figure 3.8). Microtubules are made up of 3-6 pairs of anti-

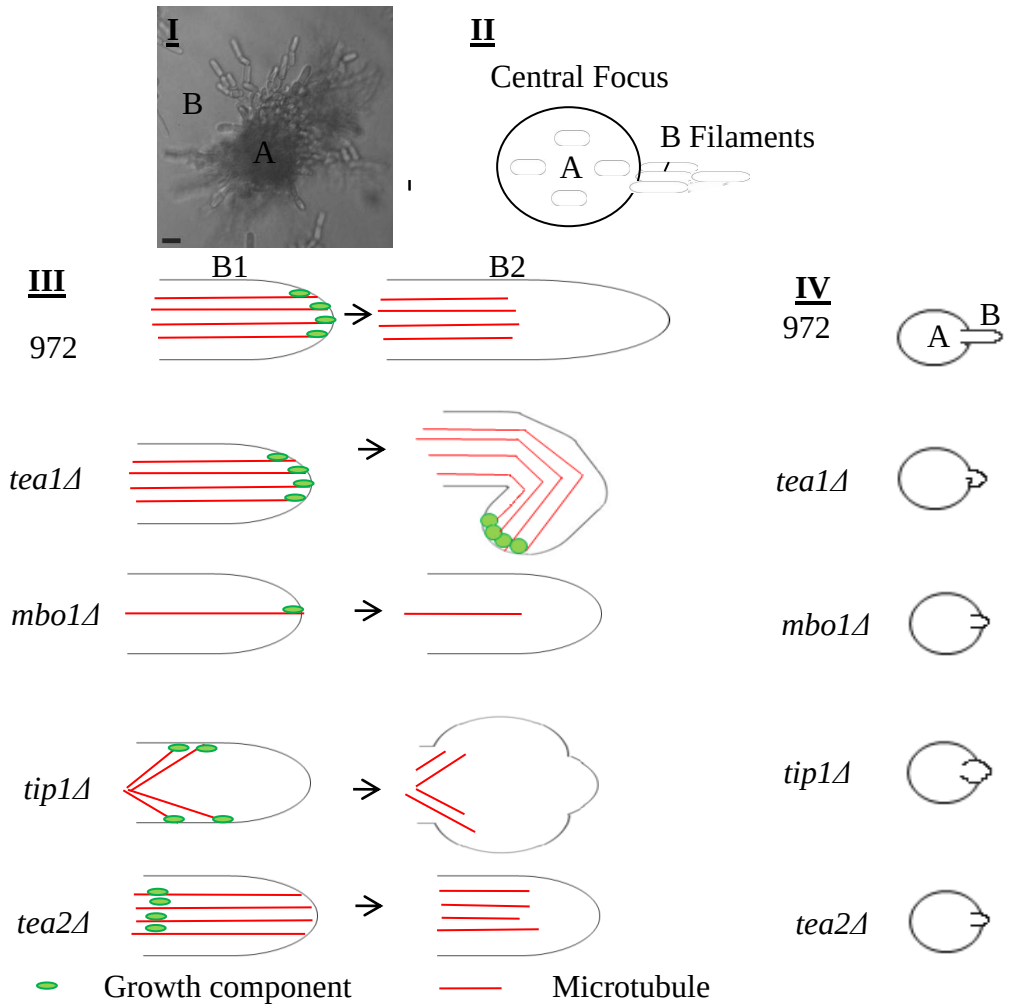


Figure 3.8 Potential causes of in-ability to form filamentous protrusions of *tea1Δ, tip1Δ, tea2Δ, mbo1Δ*. **I)** Filamentous growth of *S. pombe* 972. **II)** Schematic of filaments protruding out from central focus. **III)** Schematic of filament formation in 972 *tea1Δ, tip1Δ, tea2Δ* and *mbo1Δ*. 972; microtubule delivery of growth components to tip (B1) facilitates cellular elongation. After delivery microtubule shrinkage and catastrophe (B2) prevents excess growth component delivery. In *tea1Δ* shrinkage does not take place so microtubules curve at the tip of the cell potentially forcing cellular growth to curve too. In *mbo1Δ*, there are not enough microtubules for efficient delivery of growth components therefore no cellular elongation. In *tip1Δ* growth components may be delivered to medial regions of the cell. In *tea2Δ* the microtubules reach cell ends, but growth component delivery does not occur. **IV)** Effect of aberrant filament formation on invasive focus of 972 *tea1Δ, tip1Δ, tea2Δ* and *mbo1Δ*. 972; Efficient delivery of growth determinants allows filament formation. *tea1Δ, tip1Δ, tea2Δ* and *mbo1Δ;* filament formation is blocked due to in-efficient growth site determination.

parallel β sheets and originate from microtubules organizing centres (MTOC) (Hagan 1998). Mto1 is involved in establishing MTOCs (Samejima et al. 2005). Cells lacking Mto1will be unable to form as many microtubules and so there will be reduced delivery of growth constituents to cell ends resulting in lack of monopolar growth and filamentous protrusions (figure 3.8).

Microtubules are dynamic structures which undergo rounds of growth and catastrophic collapse. Microtubules grow out towards the cell tip and when they reach it they depolymerise, a process known as catastrophe (Drummond et al. 2000). Tip1 ensures that they only undergo catastrophe when they reach the cell tip as opposed to the cortical wall (Busch et al. 2004). Cells lacking Tip1 undergo premature microtubule catastrophe and so the growth constituents are not delivered to the cell tip. This means monopolar growth is not initiated in the correct position and so filamentous protrusions do not form resulting in exhibition of an aberrant phenotype (figure 3.8).

Individual analysis of these strains has implicated specific functions of their represented proteins within the tertiary stage of invasion, but what does the consolidated analysis of these strains suggest about filament formation?

3.3.4ib: Monopolar growth may be unique to the tertiary stage of invasion

The ability of *tea1Δ, tea2Δ, tip1Δ* and *mbo1Δ* to complete the secondary stage of invasive growth but not the tertiary, suggests that efficient microtubule mediated growth site determination is not essential for the secondary stage. This may be because only the tertiary stage follows strict monopolar growth. Once cells have penetrated the agar (secondary stage), the cells may still grow in a bipolar pattern and the direction of growth is not important. Bipolar growth may continue until a critical size of the central focus has been reached which then causes activation of monopolar growth of peripheral cells and results in the tertiary stage of the invasive process, filament formation (figure 3.9). *Tea2Δ, tip1Δ, tea1Δ* and *mbo1Δ* all confer miss-positioning of growth site but growth still takes place, albeit with a mutant morphology. This mutant morphological growth would still result in overall focus growth to reach the threshold size for induction of monopolar filamentous growth. However, monopolar growth requires strict control of growth site determination, as facilitated by efficient microtubule associated proteins, therefore *tea2Δ, tip1Δ, tea1Δ* and *mbo1Δ* are not able to initiate this monopolar growth form resulting in a lack of formation of filamentous protrusions (figure 3.9).

972

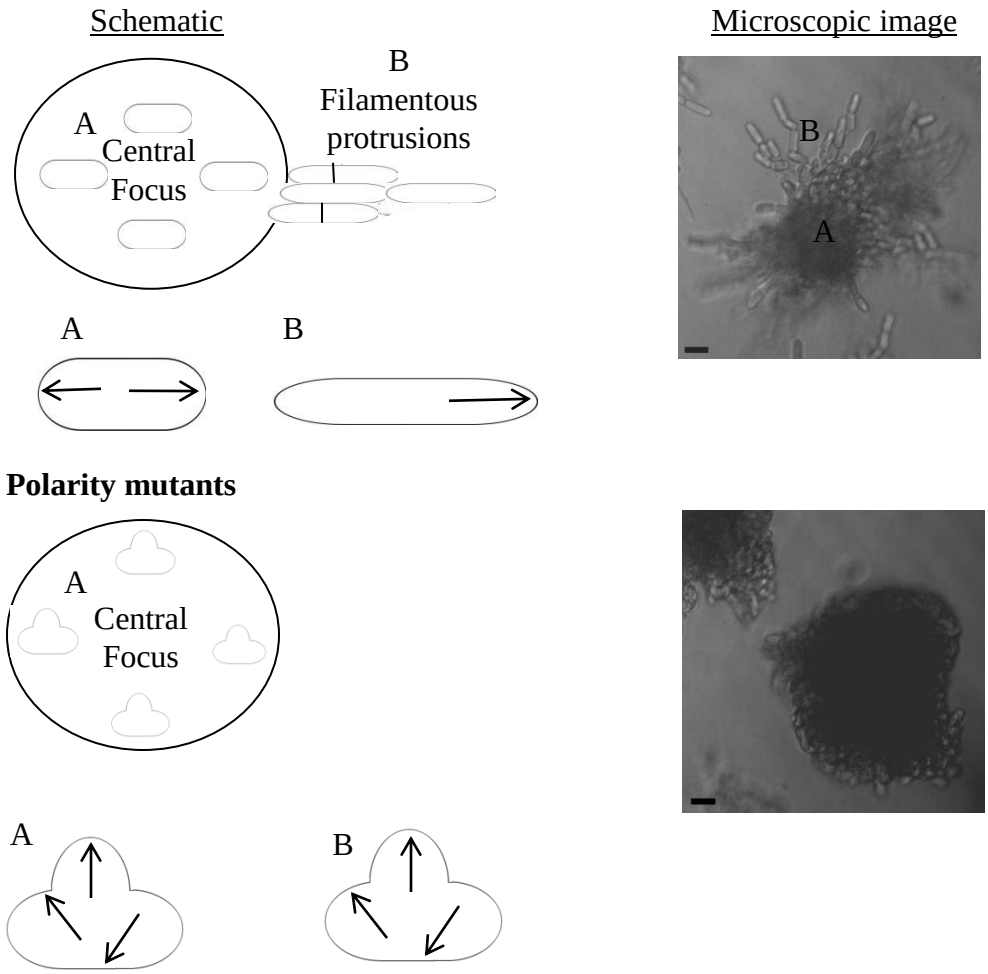


Figure 3.9 Monopolar growth may be restricted to the tertiary stage of the invasive process. **Schematic and microscopic image of 972:** In the central focus, A, cells may grow in a bipolar manner until a threshold focus size is reached. This induces the formation of filamentous protrusions (B) which requires a switch to monopolar growth. **Schematic and microscopic image of polarity mutants:** In the central focus (A) cells grow with mutant morphology as a result of miss- positioned growth sites this still enables a threshold focus size to be reached and the induction of monopolar growth, however the mis-positioning of growth site means monopolar growth is not successful and filamentous protrusions are not formed.

Tea2, Tip1, Tea1 and Mbo1 have well-defined roles in growth site determination which highlights the importance of this process in the tertiary stage of invasive growth. The determination of growth site may also be attributed to the functional roles of Sppc18.10 and Alg10.

3.3.4ic: Alg10 , Spcc18.10 may function in growth site determination

Alg10 is glycosyl-transferase that is involved in N- linked glycosylation (http://old.genedb.org/genedb/pombe/), a form of posttranslational modification that takes place at the endoplasmic reticulum (ER). This protein is responsible for the addition of the final glucose to the precursor used for the N-glycosylation of many proteins (Kelleher et al. 2001). It is the rate limiting step in the process. Adhesins on the cell surface are highly dependent on glycosylation for their function. Potentially cells lacking Alg10 are not able to glycosylate specific cell surface proteins that are required for filament formation. The glycosylated proteins may enable cell-cell communication to determine their localisation with respect to the external environment. Signal transduction could then induce monopolar growth from the appropriate site that will form filamentous protrusions into the external environment and away from the central focus. As Alg10 is responsible for the rate limiting step in the N-glycosylation process it could be that it is the number of N-glycosylated proteins on the surface and not the presence of a specific one that causes this lack of filament formation.

The *S.cerevisiae* orthologues of Alg10 and Spcc18.10 (see below) are involved in bud-site selection (Casamayor et al. 2002). Strains defective in *Sc.ALG10* showed a mutant budding pattern, a phenotype shared by the *Sp.*Spcc18.10c *S. cerevisiae* orthologue. The budding process in *S. cerevisiae* determines the new site of cell growth. This could be similar to the process taking place in *S. pombe* during the tertiary stage of invasive growth, as cell growth has to protrude away from the central focus. Potentially, the inability to form filamentous protrusions arises from the incorrect position of growth site, as described for the cell polarity proteins in figure 3.9. If the site was miss-positioned, the filamentous protrusions formed may extend back into the focus rather than out and away from the focus therefore exhibiting the Class Ia phenotype.

The tertiary stage of invasion appears to be dependent on the selection of appropriate growth site. The remaining strains (*asl1Δ, kes1Δ*and *fkh2Δ*) that were unable to form filamentous protrusions may be able to establish the site of growth but not be able to grow once it has been established.

3.3.4id: Changes in cell wall composition may be required for filament formation

Fission yeast cell walls are made of 1-3 beta glucans, with 1,6 beta glucan side-chains (Ribas et al. 1991). Most cell wall proteins are GPI anchored to 1-6 β glucans, but a small number form alkali-sensitive linkages to 1-3 β glucans (de Groot et al. 2007). Asl1 (alkali sensitive linkage 1) is a putative glucosyl hydrolyse and a 1-3 β glucan bound cell wall protein. Cell wall proteins are involved in growth and division of the cell in response to environmental changes. Asl1 could be involved in re-organisation of the cell wall via its glucosyl hydrolase activity which cleaves glucans. Psu1 is the only other ASL cell wall protein in *S. pombe* and it is essential (http://old.genedb.org/genedb/pombe/) therefore the effect on invasive morphology has not been studied. The inability of *asl1Δ* to form filamentous protrusion could arise from lack of changes in cell wall composition that are specifically required for monopolar growth and filament formation. This suggest that filament formation is facilitated, in part, from an alteration of the cell wall properties.

Kes1 is an oxysterol binding protein that is involved in ergosterol biosynthesis, which is a major component of fungal cell membrane. This function is based on homology to *S. cerevisiae* KES1 which is attributed with the GO biological process of "maintenance of cell polarity, exocytosis and endocytosis". *Sc*KES1 is thought to directly affect *Sc*.CDC42 localization via its role in vesicular transport (Kozminski et al. 2006). Vesicle trafficking is important in bud formation as it delivers growth facilitating vesicles to the sites of growth (Kozminski et al. 2006). If *Sp*.Kes1 has a similar role in vesicular transport, the inability to from filamentous protrusions may arise from an inability to deliver growth facilitating vesicles or Cdc42, to the site of monopolar cell extension. Cdc42 activity has already been discussed with regards to the secondary stage of invasion. The phenotype displayed by *kes1Δ* suggests Cdc42 regulation is also important during filament formation.

Filamentous cells undergo a monopolar growth pattern and exhibit an elongated morphology. The determination of growth site orchestrates the monopolar growth pattern exhibited by filamentous cells. As yet the elongation of these cell has not been considered. Two strains (*kes1Δ*and *fkh2Δ*) that were unable to form filamentous protrusions suggests this elongation is a result of delay in cytokinesis.

3.3.4ie: A delay in cytokinesis may be required for cellular extension in the tertiary stage of invasion

Fkh2 is a fork head transcription factor that inhibits expression of many genes required for cytokinesis. Therefore, cells lacking Fkh2 will undergo premature cytokinesis (Agarwal et al. 2010). Potentially this may result in an inability to form filamentous protrusions as the cells cannot elongate out from the central focus as they are continually going through premature cytokinesis (figure 3.10). This implicates temporal control of cytokinesis during the tertiary stage of the invasive process. The other transcription factor in control of the cytokinetic wave of gene expression is Sep1, which induces up-regulation of gene expression. *Sep1Δ* strains exhibit elongated filamentous cells (section 3.11.3, table 3.9) strengthening the argument for cytokinetic control during filament formation.

The *C. albicans* FKH2 homologue is required for hyphal formation and the *Ca.FKH2*Δ exhibits a similar phenotype as it is unable to form filamentous colonies (Bensen et al. 2002). This suggests conservation of Fkh2 functional role between *S. pombe* and *C. albicans.*

*Sp*Kes1 localises to the dividing septum (http://www.riken.jp/SPD/36/36H03.html) therefore may be involved in control of cytokinesis and coordination of cellular extension.

Analysis of the Class Ia strains has so far focused on physical growth during the tertiary stage of the invasive process, without any consideration of the signalling events. Analysis of Spcc18.10 highlights a potential signalling compound of the tertiary stage of growth.

3.3.4if: Potential signalling compounds in the tertiary stage of the invasive process

Spcc18.10Δ represents a pyridoxine-pyridoxal-pyridoxamine kinase (http://old.genedb.org/genedb/pombe/). This kinase functions to salvage the active form of vitamin b6 (pyridoxal-5-phosphate, P-5-P) from nutrients taken up from the growth substrate (Yang et al. 1998). It is responsible for the phosphorylation of the P-5-P precursors pyridoxal, pyridoxamine and pyridoxine, each differing in the functional group present on the 4 position of the pyridine ring, an aldehyde, amino and hyroxymethyl group respectively (Ike J. Jeon 1995). Pyridoxine and pyridoxamine then require a further oxidation step before they are fully converted to active P-5-P. P-5-P is

972

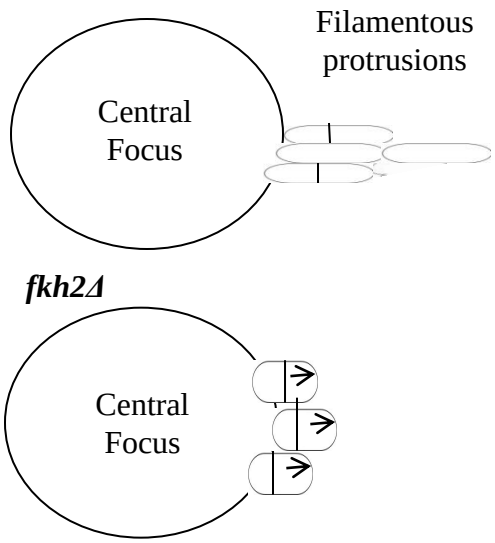


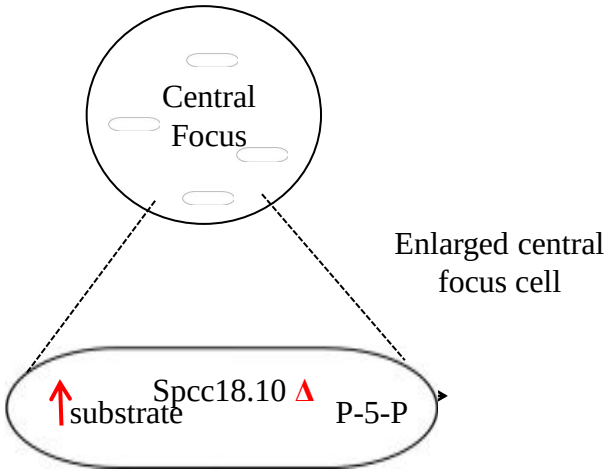
Figure 3.10 Premature cytokinesis restricts formation of filamentous protrusions. **972:** Filamentous protrusions radiate out from the central focus. ***fkh2Δ:*** filamentous protrusions may not be able to form as premature cytokinesis prevents cellular elongation.

involved in many stages of amino acid and cellular metabolism, where it functions largely as a co-enzyme (Chumnantana et al. 2001). Spcc18.10c function in the tertiary stage of invasive growth could be due to production of P-5-P as a signalling compound, or as a co-enzyme. P-5-P has been shown to be excreted by *S. pombe* (Chumnantana et al. 2001). A high cell density facilitates invasion (Dodgson et al. 2009). This may be because there are more cells producing a stimulant for filament formation, such as P-5-P.

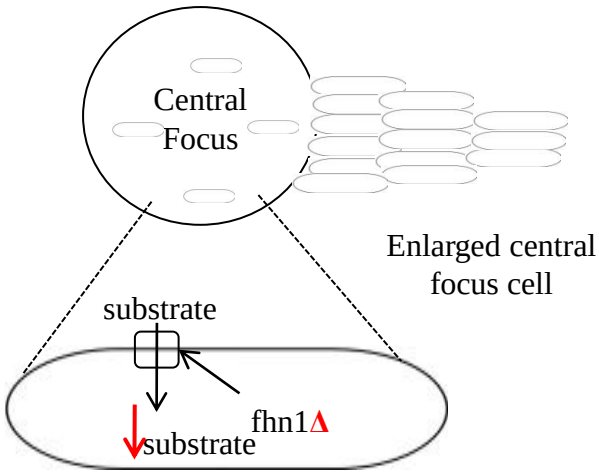
Alternatively the phenotype exhibited by *spcc18.10Δ* could arise from an inhibitory effect of substrate accumulation. Spcc18.10 phosphorylates pyridoxine, pyridoxal and pyridoxamine, therefore in *spcc18.10Δ* these compounds may accumulate. Potentially, this could lead to inhibition of filament formation. *S. pombe* actively excretes pyridoxal, pyridoxine and pyridoxamine from the cell (Chumnantana et al. 2001). These can then be assimilated into the cell by a transporter (Stolz et al. 2005). The strain representing the pyridoxine, pyridoxal, pyridoxamine transporter, *bsu1Δ*, did not exhibit an aberrant phenotype in the screen, however there is residual assimilation when this gene is deleted (Stolz et al. 2005). Fhn1 organises transporters on the cell surface (Loibl et al. 2010). As the substrates are assimilated by a transporter, potentially, in *fhn1Δ* cells there may be less substrate assimilation. This is in contrast to *spcc18.10Δ* cells where there is presumable excess substrates as there is no enzyme to convert them to P-5-P. *fhn1Δ* exhibited a thickened filamentous rope structures (Class Ib, table 3.8), which may be caused by excess filamentous protrusions. This suggests the substrates could be involved in negative regulation of filament formation (figure 3.11). The cells on the periphery of the focus would not assimilate as much substrate as the central focus cells as they are not surrounded by as many cells. This reduced level of substrate in the peripheral cells may allow filament formation by an as yet undetermined mechanism.

Filament formation may involve cell surface adhesins, which are also involved in flocculation. *Plr1Δ* has been described as highly flocculent after prolonged stationary phase growth (Morita et al. 2004). Plr1 converts pyridoxal to pyridoxine, therefore in *plr1Δ* cells there is a low pyridoxine concentration, but a higher pyridoxal concentration. This may suggest that pyridoxine negatively regulates flocculation, which strengthens its potential role in negative regulation of filament formation but suggests there are differential affects between the Spcc18.10 substrates. However,

Spcc18.10cΔ



fhn1Δ



PN symporter

Arrows indicate an increase in decrease in cellular Spcc18.10 substrate levels in comparison to 972 in the mutant (Δ)stated

Figure 3.11 Potential role of Spcc18.10 substrate during filament formation. Spcc18.10 substrate intracellular concentration is dependent on assimilation and conversion rate. ***Spcc18.10Δ:*** Spcc18.10 substrate assimilation is normal, but intracellular Spcc18.10 substrate concentrations are high as Spcc18.10 is not present to convert the substrates to P-5-P. These cells are unable to form filamentous protrusions. ***Fhn1Δ:*** Substrate conversion rate is normal but substrate assimilation is low as Fhn1 is a transporter organiser so there will probably be less substrate transporter present at the cell membrane in *fhn1Δ* cells. These cells display thickened rope structures that may arise from excess filament formation. This suggests a negative role for the substrates in regulation of monopolar growth.

plr1Δ did not display a mutant phenotype in the screen. This suggests different pyridoxal derivatives could have different roles in expression of specific cell surface adhesins. If this were true, the negative regulation of flocculation by pyridoxine may cause expression of cell surface adhesins that are specifically required for flocculation but are not involved in filament formation.

Fkh2 has an FH domain, which confers the ability to bind phospho-proteins and thereby be a target of a phospho-relay signal transduction cascade (Bulmer et al. 2004). Fkh2 could be at the end of a phospho-relay cascade, initiated by cAMP/PKA signalling, which results in Fkh2 activation and inhibition of cytokinesis thereby facilitating cellular elongation and filament formation. In *fkh2Δ* cells there would be no inhibition of cytokinesis, therefore no cellular elongation which would result in an inability to form filaments (figure 3.10).

3.3.4ig: Conclusion from class Ia

Analysis of the Class Ia strains that were unable to form filamentous protrusions implicates some crucial processes in the tertiary stage of invasion:

1) The ability of *tea1Δ*, *tea2Δ*, *mbo1Δ* and *tip1Δ* to complete the secondary but not tertiary stage of invasion suggests monopolar growth is unique to the tertiary stage.

2) Positioning of the site for cellular extension may be a result of a combined effect of Tea1, Tea2, Mbo1, Tip1, Alg10 and Spcc18.10c.

3) Growth may then be facilitated at these specific sites by cell wall re-organisation.

4) The elongated morphology exhibited by filaments may be a result of delayed cytokinesis by Fkh2 activation, through a FH domain.

Class Ia strains are blocked at the tertiary stage of invasion. Strains that exhibit a Class Ib phenotype were able to form the filamentous protrusions but the rope structures were thickened. This implies the tertiary stage is de-regulated in class Ib therefore analysis of this group may provide insights into the regulation of the tertiary stage of growth.

3.3.4ii: Class Ib) Strains had thickened filamentous rope structure

The leading filament cell elongates via monopolar growth, however the cells behind this leading filament cell revert to yeast-phase, bipolar growth (Dodgson et al. 2010). Strains in class Ib appear to have more cells forming the rope structures behind the leading filament cell (arrow in figure 3.5), giving a thickened appearance. This could be due to

excess growth after the reversion to yeast-phase growth, or it could be due to excess formation of filamentous protrusions in neighbouring cells (figure 3.12). These two scenarios are both results of deregulated filament formation therefore individual analysis of the strains characterised with this phenotype may provide insights into the regulation of the tertiary stage.

3.3.4iia: Potential signalling pathways in regulation of filament formation

Three of the strains represent proteins involved in sexual development: Pac2, a transcription factor, SPAC1093.03, a phosphatase and Hse1, a protein required for correct forespore membrane morphology (http://old.genedb.org/genedb/pombe/; (Onishi et al. 2007). The presence of the different stages of sexual development control in this group suggests there may be regulatory overlap between the two pathways.

Pek1 is a MAPK kinase in the cell-integrity MAPK (Mkh1-Pek1- Pck1) pathway (Sugiura et al. 1999). This MAPK cascade is involved in cell wall construction, morphogenesis and cytokinesis (Sugiura et al. 1999). The inability of *pek1Δ* to properly regulate the tertiary stage of invasion implicates the cell-integrity MAPK pathway in regulation of cell morphology during filament formation. As the phenotype may result from an excess reversion to yeast-phase growth, Pek1 may be a negative regulator of this process. Neither Mkh1 nor Pck1 exhibited a phenotype in this screen, suggesting Pek1 may function independently of these proteins during filament formation.

Analysis of the potential regulatory signalling components suggests an involvement of mating and cell integrity MAPK signalling. Further analysis of strains exhibiting this phenotype may prove insightful to the physical changes that occur in this de-regulated growth form.

3.3.4iib: Alteration of microtubule and cell wall components results in de-regulated filament formation

Mal3 and Tea4 are both microtubule associated proteins. Mal3 is involved in maintaining microtubule integrity; it localises to the plus end of microtubules where it prevents premature catastrophe (Busch et al. 2004). Tea4 is a microtubule associated cell polarity determinant. It is capable of binding protein phosphatase 1 and so could be a mediator of a phospho-relay signal (Tatebe et al. 2005). The growth pattern within this phenotypic group could be due to excess growth in the peripheral yeast-phase cells. The

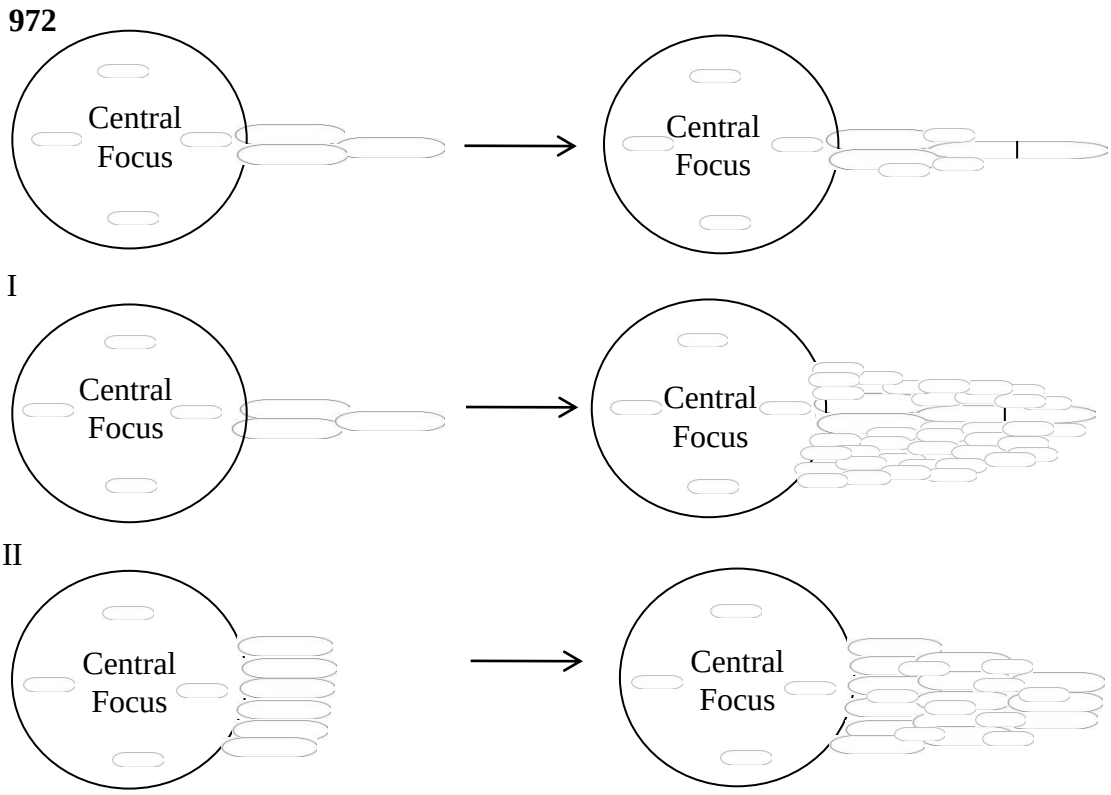


Figure 3.12 Potential modes of formation thickened filamentous rope structure in class Ib strains. *S. pombe* **972:** Filaments protrude out from the central focus and a limited number of cells revert back to yeast phase growth. **I:** Filaments protrude out from the central focus as seen for *S. pombe* 972 but following the reversion to yeast phase growth there is excess growth of these peripheral cells, leading to a thickened rope appearance. **II:** A larger number of filamentous cells protrude out from the central focus, leading to a thickened appearance. The cells that do protrude revert back to yeast phase growth at a equal level seen in *S. pombe* 972.

exhibition of this phenotype by *mal3Δ* could arise from the delivery of growth constituents to the sides of cells, due to instability of the microtubule complex, resulting in lateral growth of multiple cells and the thick rope structures that are seen. Mal3 functions alongside Tip1. *Tip1Δ* cells are unable to form filamentous protrusions. This suggests Tip1 function in microtubule stability is more important than Mal3 function during filament formation. This implies regulation of the tertiary stage of invasive growth requires strict control growth site determination.

Spn3 functions in cytokinesis, which has already been implicated in regulation of filament formation (section 3.11.1v). There are 6 other septins in the *S. pombe* genome and their representatives were not identified as morphologically aberrant from the screen. This implies an invasion-specific role of Spn3. Spn1-4 form a hetero-oligomeric ring structure at the site of cell cleavage and are thought to be important for the delivery of endoglucanases to the division septum (An et al. 2004). Control of cytokinesis appears to be important during the tertiary stage of invasive growth so perhaps Spn3 functions as the effector of this control. If this phenotype is caused by excess yeast-phase growth of the peripheral cells it implicates Spn3 as negative regulator of this process.

Acp1 is an F-actin capping protein which prevents association or dissociation of globular actin monomers (G-actin) to the barbed ends of filamentous actin (F-actin) (Nakano et al. 2006). In *acp1Δ* cells there is an accumulation of actin patches as there is nothing to prevent actin association. This implies regulation of actin-patch-mediated growth is more important for the peripheral yeast-phase cells than the leading filament cell and regulation of this growth form facilitates efficient tertiary stage invasive growth.

Fhn1 is a plasma membrane organisation protein involved with organising areas of the membrane that are symporter rich. These are referred to as MCC (Membrane Compartment of arginine permease Can1 –due to the first protein identified within this protein rich region) (Malinsky et al. 2010). This suggests tight regulation of cell wall composition and organisation are required for efficient filament formation.
The irregular tertiary stage of invasion displayed by the *mal3Δ, tea4Δ acp1Δ, spn3Δ,* and *fhn1Δ* strains suggests tight regulation of microtubules, actin patches and cytokinesis is required for efficient regulation of filament formation.

Filament formation involves cellular elongation. The Class IIa strains exhibit an extreme form of this phenotype therefore functional analysis may highlight regulatory processes for this aspect of the tertiary stage of the invasive process.

3.3.4iii: Class IIa) Strains that had elongated filamentous cells

There were nine strains that exhibited elongated filamentous cells. Individual analysis of these strains may identify how this is controlled.

3.3.4iiia: Stress-activated MAPK signalling cascade may be down regulated to achieve cellular elongation during filament formation

Wis1Δ, win1Δ and *sty1Δ* represent the stress-activated mitogen activated protein kinase cascade (SA MAPK) (Samejima et al. 1997). Wis1 and Win1 function in a phosphor-relay cascade resulting in Sty1 activation, therefore the phenotype exhibited by *wis1Δ* and *win1Δ* is presumably a direct result of loss of Sty1 activation. This signalling cascade is normally activated under conditions of high stress such as osmotic or nutrient deprivation and heat (Samejima et al. 1997). *Wis1Δ, win1Δ* and *sty1Δ* single cells exhibit an elongated morphology but only under stressful conditions when the signalling cascade is active. This suggests this signalling cascades is active during filament formation. This correlates with the screen utilising low nitrogen to induce invasive growth as this would cause activation of the stress-activated MAPK cascade.

MAPK cascades transduce signals from the environment to transcriptional control machinery via multiple rounds of phosphorylation (Soto et al. 2007). The transcription factors that SA MAPK normal transduces to (Atf1, Pcr1, Pap1) (Dunand-Sauthier et al. 2005) are b-zip transcription factors and none of these exhibited an aberrant phenotype in the screen. However, a zinc finger protein transcription factor (SPBC1105.14, table 3.9) also exhibited elongated filamentous cells, suggesting it could be a new target for this signalling cascade during filament formation.

The elongated morphology of these mutants may be due to delayed G2/M transition as Sty1 induces this transition under stressful conditions (Shiozaki et al. 1995). G2/M transition is controlled by Cdc2 (Nurse et al. 1980). Cdc2 is a phospho-protein subject to inhibitory phosphorylation by Wee1 and activation by de-phosphorylation by Cdc25 (Russell et al. 1987; Millar et al. 1991). During cellular stress Sty1 mediates control of Cdc2 via Plo1. Active Sty1 phosphorylates Plo1, which facilitates recruitment to the spindle pole body where it controls G2/M transition via Cdc25/Wee1 activity (Nunez et

al. 2010). In *sty1Δ* cells induction of mitosis does not take place and cells are stuck in G2 thus exhibiting an elongated phenotype.

The G2/M delay caused by Sty1 deletion may be representative of cellular elongation required during the tertiary stage of invasive growth. Perhaps the activated Sty1 pathway is down regulated to induce G2/M delay and thus cellular elongation during filament formation. The delay of G2/M may also be the cause of the elongated filamentous cells in *cpc2Δ.*

3.3.4iiib: Regulation of Wee1 mRNA levels may facilitate filament elongation

Cpc2 elicits cellular responses via alteration of mRNA translation (Shor et al. 2003) and therefore protein level. The exhibition of an elongated phenotype by *cpc2Δ* implies that this form of protein regulation is used in filament formation. Specifically it has been shown to increase mRNA levels of Pyp1 phosphatase (Nunez et al. 2010). Pyp1 is the phosphatase responsible for removing phosphate from and thus inhibiting Sty1 (Samejima et al. 1997). This idea appears contradictory to the above proposed Sty1 role in filament formation, where a decrease in Sty1 activity may cause cellular elongation. Deletion of Cpc2 would cause a decrease in Pyp1 activity and subsequently an increase in Sty1 activity. However, if the proposed model is correct and Sty1 is down regulated in invasively growing cells to cause elongation during filament formation then the Cpc2 deletion would have no effect as there would be less Sty1 for Pyp1 to inhibit. In this scenario the elongated phenotype could be caused by an alternative Pyp1 independent effect of Cpc2. Cpc2 also down-regulates Wee1 mRNA translation under cellular stress (Nunez et al. 2010). Wee1 is responsible for the phosphorylation and inactivation of Cdc2 (Russell et al. 1987), thus repressing mitotic entry. *Cpc2Δ* single cells exhibit an elongated morphology as there is more Wee1 being translated (as Cdc2 normally down-regulates the level of translation). This inhibits mitosis resulting in pro-longed G2 and an elongated morphology. As with the *sty1Δ, wis1Δ* and *win1Δ* phenotype, the regulation of Cpc2 activity may be involved in controlling cellular elongation during filament formation. This is most probably mediated via its effect on Wee1 causing pro-longed G2. Cpc2 is regulated by Pck2 (Won et al. 2001) which is activated by Rho1 (Arellano et al. 1999). Rho1 signalling has already been implicated as a negative regulator of the invasive processes (section 3.3.3ivc). The exhibition of an elongated phenotype by *cpc2Δ* suggests this regulation includes control of filament formation.

Rgf1Δ, which also exhibited an elongated phenotype, encodes another protein related to Rho1 signalling.

3.3.4iiic: Potential roles of Rgf1 during filament elongation

Rgf1 is an activating protein (guanine nucleotide exchange factor- GEF) for Rho1 (Garcia et al. 2009). The Rho1 signalling cascade has already been implicated in invasive growth by the Rho1 GAP, Rga8, Wsc1 (section 3.3.3iv) and Cpc2 (above). Rho1 signal is also transduced via Pck2 (Arellano et al. 1999). Pck2 has been shown to activate the cell-integrity pathway as well as interact with Cpc2 (Won et al. 2001). *Cpc2Δ* also displayed an elongated filamentous phenotype therefore it seems likely the Pck2 –Cpc2 mediated Rho1 signalling cascade is important in filament formation. Finally, Rho1 is also involved in cell wall biosynthesis via activation of β glucan synthase, which is responsible for production of the major constituent of cell walls, β glucan (Arellano et al. 1996). Presumably, *rgf1* deletion would cause a decrease in Rho1 activity and less β glucan synthesis. This is similar to *wsc1*Δ suggesting that a decrease in β glucan synthesis is associated with an increase in invasive efficiency. There are 2 GEFs for Rho1: Rgf1 and Rgf2. These are thought to be redundant (Mutoh et al. 2005). However, *rgf2*Δ did not exhibit an aberrant phenotype in the screen. This is quite a dramatic result as previously Rgf1 and Rgf2 have been shown to function identically. This implicates a Rgf1specific role during filament formation.

The analysis of Wis1, Win1, Sty1, Cpc2 and Rgf1 has led to the proposal of mechanisms that may cause filament elongation during the tertiary stage of the invasive process. The morphology exhibited by these strains suggests these proteins may be down regulated to cause cellular elongation during invasion. In contrast, Sep1, is probably not involved in regulation of filament formation as the aberrant phenotype displayed does not resemble the elongated morphology of the leading filament.

3.3.4iiie: Sep1 is probably not involved in control of filament elongation

The morphology of *sep1Δ* differs to that of the other class IIa strains (figure 3.5). Sep1 is a fork head transcription factor that controls cytokinesis (Ribar et al. 1999). Its morphology reflects this; the cells appear unable to complete cytokinesis. This morphology is also exhibited in single cells. Unlike the other strains of this phenotypic group, the morphology of *sep1Δ* does not suggest that Sep1 could be down regulated to

elicit elongation during filament formation. It seems more likely this phenotype is an exacerbation of the single cell phenotype and does not represent mechanisms of control during the tertiary stage of invasion.

This concludes the analysis of individual strains exhibiting the class IIa phenotype. The final point of analysis for the Class IIa strains is based on a similarity in phenotype displayed by treatment of *S. pombe* 972 with a cAMP analogue.

3.3.4iiif: Class IIa phenotype is similar to a cAMP-induced phenotype

Prevorovsky *et al*. (2009) describe the invasive filaments of *S.pombe* as elongated when treated with excess cAMP analogue. This morphology is similar to that displayed by this phenotypic group (excluding *sep1*Δ) which implies these two phenotypes may be related. cAMP signalling has been proposed to down-regulate the Sty1 MAPK pathway (Stettler et al. 1996). Therefore, the phenotype displayed upon treatment with excess cAMP analogue could also result from a loss of Sty1 function as is seen in *sty1Δ.*The cAMP signalling cascade is required for induction of invasion by low nitrogen (Amoah-Buahin et al. 2005). In those low-nitrogen conditions the Sty1 pathway will also be active. The regulation of the Sty1 pathway by cAMP may cause the cellular elongation required for filament formation. Additionally, the Sty1 pathway is required for induction of the mating response, which is also stimulated by low nitrogen. Therefore the inhibition of Sty1 by cAMP during invasion may also act to inhibit the mating response.

Analysis of the class IIa phenotypic group suggests that cellular elongation during filament formation may be a result of a delayed G2/M transition facilitated by down regulation of the stress-activated MAPK, and the Rho1 signalling cascades.

The final phenotypic group identified in the screen displayed erratically structured filamentous cells. Individual analysis of these strains may highlight further regulatory processes during filament formation.

3.3.4iv: Class IIb) Strains that had erratically structured filamentous cells

Strains that exhibited this phenotype were able to form filamentous protrusions but the constituent cells were not uniform in shape or size. Cells in these filaments were erratic in shape and size, though this is not true for every cell within the filament; some are

normal in size and morphology. This phenotypic group is caused by aberrations in single cell structure within the filaments therefore regulation of filamentous growth may share features with regulation of single cell growth.

3.3.4iva: Filament and single cell morphology share regulatory processes

Class IIb phenotypic group is a heterologous mix of strains, some of which exhibited the aberrant morphologies specifically during invasive growth and some that exhibited the phenotype during single cell growth too (figure 3.6). This implies that certain aspects of morphological regulation are unique to invasive growth, but some are shared between the two growth forms. A morphological screen of all the class IIb strains as single cells would identify which strains exhibited invasion specific aberrations. This was not performed here due to time constraints.

Individual analysis of the strains that represent potentially interesting processes may highlight the regulatory mechanisms involved during filament formation.

3.3.4ivb: Control of cytokinesis facilitates efficient filament formation

Various defects in the cytokinetic process results in erratically structured filamentous cells. Defects in transcriptional control occur in *spt8Δ, ace2Δ* and *sep11Δ* and defects in check-point control occur in *rad24* and *rad25* (http://old.genedb.org/genedb/pombe/). Spt8 facilitates chromatin remodelling via histone acetylation, which opens up the DNA structure facilitating entry of transcriptional machinery (Batta et al. 2009). Spt8 then acts as to recruit transcription factors to TATA boxes within gene promoters thus facilitating cytokinetic gene expression. Ace2 and Sep11 are responsible for transcriptional regulation of many cytokenetic genes (Alonso-Nunez et al. 2005). Ace2 also exhibits a hyper-invasive phenotype, showing deregulation of cytokinesis results in loss of control of invasive growth at the secondary and tertiary stages of the process. Rad24 and Rad25 are DNA checkpoint proteins that have been shown to be involved in regulation of cytokinesis (Ishiguro et al. 2001; Mishra et al. 2005). They are 14-3-3 proteins , which are known to be involved in several signalling cascades. Their phenotypes probably arise from effects on the cytokinesis checkpoint.

The regulation of filament morphology is not restricted to transcriptional mediated control of cytokinesis. Defects in regulation of cytokinesis during the contraction of the

actomyosin ring occur in *rlc1Δ* and *mid2Δ* and these also result in erratically structured filamentous cells. Rlc1 is a myosin regulatory light chain for Myo2 which is the motor protein involved in actomyosin contractile ring constriction (Sladewski et al. 2009). Mid2 is proposed to function alongside Sep1-4 resulting in functional actomyosin ring formation (Wu et al. 2010) though it is not directly involved in the establishment of the actomyosin ring. Mid2 is under control of Ace2 which is the master transcription factor in control of cytokinesis (Petit et al. 2005). The *mid2Δ* single cell phenotype is elongated and multi-septate cells. This is not the same phenotype seen in filaments, where cells complete cytokinesis. This suggests a separate function during filament formation. Finally *cyk3Δ* represents a protein involved in cytokinesis (http://old.genedb.org/genedb/pombe/), though as yet the exact role is unclear. Deletion of the *S.cerevisiae* orthologue confers a non-invasive phenotype (http://www.yeastgenome.org), showing that although there may be similarities between the invasive process of the two yeasts there are also differences.

The exhibition of this phenotype by these cytokinesis-defective strains suggests morphology of filamentous cells is in part regulated by control of cytokinesis. Control by cellular growth machinery also contributes to the uniform morphology of filamentous cells.

3.3.4ivc: Regulation of cellular growth machinery contributes to normal filament morphology

Regulation of cellular growth pattern contributes to filament morphogenesis as represented by defects in *psd1Δ, asp1Δ, spac637.06Δ, gpt1Δ, end4Δ, ucp8Δ* and *wsc1Δ*. Psd1 is proposed to control spatial organization of cytokinesis and is one of three *psd* genes whose products are responsible for the majority of cellular phosphatidlyetholamine (PE) production; a major component of cell membranes (Luo et al. 2009). Interestingly Psd1 is thought to be functionally redundant with its homologues Psd2 and Psd3 (Luo et al. 2009). Psd2 is not in the deletion libraries, however Psd3 did not exhibit an abnormality in the screen. This could suggest Psd1 is specifically required for PE production during filament formation. Asp1 is an inositol hexakisphosphate kinase responsible for the generation of inositol phosphates (Feoktistova et al. 1999). It is involved in cortical actin cytoskeleton organization (http://old.genedb.org/genedb/pombe/). Single cell *asp1Δ* exhibits altered morphology and growth defects, which is similar to the morphology seen in filaments. *Asp1Δ* has

recently been described as non-invasive (Pohlmann et al. 2010) though the result from this screen is contradictory to this as *asp1Δ* did not exhibit a non-invasive phenotype. This discrepancy in phenotype could result from a number of factors. Firstly, Pohlman *et al*. 2010, used a relatively low density of cells for invasive growth compared to this screen. This laboratory has previously shown that the density of cells used for invasive growth affects invasive efficiency (unpublished data). Amt1, Amt2 and Amt3 were described by Mitsuzawa *et al*, (2006) as non-invasive at a low cell density(Mitsuzawa 2006)(Mitsuzawa 2006), but shown to invade at a high cell density by the John Armstrong laboratory (unpublished data). Secondly the media used by the Pohlman laboratory (2% agar YES) was different to the media used in this screen (1% agar LNB). This thesis has discussed the importance of agar concentration in invasive efficiency (section 3.3.1) therefore the variation media used may also contribute to the discrepancy between phenotypes.

SPAC637.06 has not yet been characterised but is proposed to be an α1,2 galactosyltransferase based on homology to *S.cerevisiae* MNN10 (http://old.genedb.org/genedb/pombe/). It is involved in cell wall biogenesis via protein glycosylation. Gpt1 is involved in 1,6 β glucan biosynthetic process and Ucp8 is involved in actin cortical patch assembly (http://old.genedb.org/genedb/pombe/) (Fernandez et al. 1996). End4 is involved in cellular growth through protein trafficking along the microtubules. Its localisation is dependent on Tea1 (Castagnetti et al. 2005). Wsc1 is a transmembrane receptor involved in organization of the actin cytoskeleton in response to stress (Philip et al. 2001).

The inability of these strains to regulate growth efficiently could result in aberrant cell morphology as displayed in class IIb.

Regulation of cytokinesis and cellular growth co-ordinate the physical aspects of morphological control. Some strains within this phenotypic group highlight potential signalling processes involved in regulation of filamentous morphology.

3.3.4ivd: Cell integrity and stress activated MAPK cascades may regulate filamentous cell morphology

Analysis of individual strains (*mak3Δ, pyp1Δ, spac3g9.05Δ* and *rnc1Δ*) within this phenotypic class implicates MAPK signalling cascades as morphological regulators. Mak3 is a histidine kinase responsible for indirect activation of Sty1, part of the stress

activated MAPK response cascade (Buck et al. 2001). *Sty1Δ* exhibited elongated filamentous cells (see section 3.11.3i) which is distinct for the phenotype displayed here. This suggests *mak3Δ* phenotype is not solely dependent on its role in Sty1 MAPK signalling. However, the Mak3 relationship with the stress activated MAPK still implicates this signalling cascade in regulation of filamentous morphology. *Mak3Δ* does not exhibit an aberrant phenotype in single cells, implying a specific role in regulation of filament morphology.

Pyp1 is the de-activating protein phosphatase for Sty1, a stress activated MAPK component (Shiozaki et al. 1995). The de-regulation of filament formation in *pyp1Δ* further strengthens the implication of stress activated MAPK during control of filament morphology. SPAC3G9.05 is a small GTPase activating protein, though this role is only inferred from homology to *S. cerevisiae* SPA2 (http://old.genedb.org/genedb/pombe/). *Sc*.SPA2 acts as a scaffold protein in the cell integrity MAPK response (van Drogen et al. 2002). The *S. pombe* orthologue could also serve this function, implicating the cell integrity MAPK in control of filament cell morphology. The *S. cerevisiae SPA2* deletion mutant is not capable of invasive growth (Jin et al. 2008). This suggests that although there are some similarities between the processes in the two yeasts they are still quite diverse.

Rnc1 is an RNA binding protein that is involved in negative regulation of the cell-integrity MAPK. Rnc1 stabilises Pmp1 mRNA, resulting in increased translation (Sugiura et al. 2003). Pmp1 is a phosphatase for Pmk1, the final effector kinase in the cell-integrity signalling cascade (Sugiura et al. 1998). Removal of the phosphate group from Pmk1 results in its inactivation. Without this negative regulation Pmk1 will be more active, which results in morphological aberrations. This implicates the cell-integrity MAPK pathway in regulation of filament morphology.

The MAPK response pathways are well described signalling cascades that alter cellular morphogenesis. Individual strains within this phenotypic class also represent some less well described potential signalling pathways.

3.3.4ive: Alternative signalling compounds during filament formation

The classification of *spac222.08cΔ* and *bit61Δ* as class IIb strains suggests the involvement of uncharacterised signalling pathways in the control of filament morphology. SPAC222.08c is proposed to be a glutamine amino-transferase subunit

which is involved in pyridoxine (PN) biosynthesis. This function is based on homology to *S. cerevisiae* SNO1 (Rodriguez-Navarro et al. 2002). PN is substrate for Spcc18.10. The presence of Spcc18.10 substrates in the cell has already been discussed as a potential morphological regulator (figure 3.11). PN may have an effect on filamentous morphology via a role as a chemical message or function as a co-enzyme. The de-regulated morphology of *spac222.08cΔ* cells further implicates PN as a regulator of the tertiary stage of invasion.

Bit61 is part of the TORC2 complex involved in the target of rapomycin (TOR) signalling cascade (Hayashi et al. 2007). *S. pombe* has two TOR proteins, Tor1 which forms TORC2 and Tor2 which forms TORC1. Interestingly this is the first time either pathway has been implicated in filament formation. This suggests TOR signalling does not play a major role in the process. The *S.cerevisiae* homologue regulates actin organisation during growth (Schmidt et al. 1996) so this protein probably has a similar role and thus its deletion results in a morphological aberration.

The proposed signalling pathways may contribute to filament morphology. The final strain for individual analysis, *gsa1*Δ, implicates control of cellular homeostasis in regulation of filament morphology.

Gsa1 is involved in glutathione biosynthesis (http://old.genedb.org/genedb/pombe/). Glutathione can be thought of as a cellular buffer for damaging oxidants from the environment (Wang et al. 1997). Gsa1 is glutathione synthetase subunit (http://old.genedb.org/genedb/pombe/), therefore when it is deleted, there is not as much glutathione produced. This would leave the cell susceptible to environmental toxins. The aberrant morphology may result from these threats.

Analysis of individual strains within this phenotypic class implicates cytokinesis and cellular growth in control of filamentous morphology which may be achieved through MAPK signalling and re-organization of actin.

This concludes the individual analysis of phenotypic classes identified from the screen. Strains that exhibited a poorly-invasive or hyper-invasive phenotype were also capable of exhibiting a morphological aberration. Strains that exhibit aberrations in more than one phenotypic group represent proteins that are involved in more than one stage of the invasive process.

3.3.4v: Strains can exhibit multiple phenotypes

Strains can exhibit an invasive efficiency (secondary stage) defect and a morphologically (tertiary stage) aberrant phenotype. This implies some processes are shared between the two stages which may be highlighted by individual analysis of *atg9Δ spac6g9.14Δ, adn1Δ pyp1Δ* and *wsc1Δ*. Atg9, is an autophagy associated protein that is involved in membrane trafficking (http://old.genedb.org/genedb/pombe/). It is not obvious what role this may play in the invasive response. Adn1 is a transcriptional regulator of cellular adhesion (Dodgson et al. 2009), the aberrant phenotype suggests primary stage associated transcriptional control is also important during the tertiary stage of the invasive process. Pyp1 and Wsc1 are both associated with the stress activated MAPK. Wsc1 is proposed to be a membrane bound receptor for MAPK activation and Pyp1 is an inhibitory phosphatase that targets Sty1, the final kinase in the SA MAPK cascade (Shiozaki et al. 1995; Philip et al. 2001). The deletion of *pyp1* and *wsc1* would be expected to produce opposite effects as one is an inhibitor and the other an activator of the SA MAPK. This antagonism is seen with respect to the secondary stage of invasion as *wsc1Δ* (activator) exhibits a hyper-invasive phenotype and *pyp1Δ* (inhibitor) exhibits a poorly invasive phenotype. However the same morphological aberration (erratically structured filamentous cells) is exhibited during the tertiary stage of invasion. SPAC6G9.14 has not yet been characterised but is proposed to be an RNA binding protein based on homology to *S.cerevisiae* Puf4 (http://old.genedb.org/genedb/pombe/). Previous work has shown that RNA binding proteins can effect MAPK signalling (Sugiura et al. 2003) so potentially SPACG9.14 may function to stabilise MAPK associated mRNA and therefore be involved in regulation of this signalling cascade.

The representative proteins of strains that exhibit secondary and tertiary stage aberrations may reflect processes that are required during both stages of the invasive process. The individual analysis of these strains suggests that the stress-activated MAPK pathway may be involved in regulation of these two stages. It may function to negatively regulate the secondary stage of invasion, but the mechanism for regulation of filament morphology is unclear, as deletion of an activator and inhibitor of the signalling cascade results in the same morphological aberration.

The screen identified 177 strains that exhibited aberrations during the invasive process. This is the first morphological screen for invasive aberrations, however previous non-

morphological screens for invasive mutants have been carried out in *S. pombe* and *S.cerevisiae.* Comparison of strains identified by the different screens may highlight conserved mechanisms during invasive growth.

3.3.5: Comparative analysis with other screens

The process of invasive growth is studied in various other organisms including *S. cerevisiae.* Previous work on this organism included an over-expression/gene disruption screen that looked for mutant invasive phenotypes (Jin et al. 2008). As the *S. cerevisiae* screen and this *S. pombe* screen both identified strains that were non-invasive, comparison of strains identified may highlight conserved mechanisms of the secondary stage of invasion.

3.3.5i: Comparison between the *S. pombe* screen and an *S. cerevisiae* over-expression screen

A large scale analysis of invasive growth of *S. cerevisiae* identified 487 genes conferring abnormal invasive phenotypes (Jin et al. 2008). These abnormal phenotypes were associated with invasive efficiency and colony morphology. This comparison is restricted to the invasive efficiency defects of the 34 strains classified as non- or poorly-invasive in the *S. pombe* screen, as the morphological aberrations addressed in the *S. cerevisiae* screen were based on colonies rather than filaments. 34 strains were classified as non-invasive or poorly invasive in the *S. pombe* screen, six of which do not have an *S. cerevisiae* orthologue. 28 of the *S. pombe* strains classified as non- or poorly-invasive had *S. cerevisiae* orthologues and 11 of these were identified in the *S. cerevisiae* screen, therefore 17 were not. This suggests the conservation of some but not all mechanisms of invasion between the two yeasts. Analysis of the orthologues that exhibited invasive defects in both screens may highlight conserved processes shared between the two yeasts.

3.3.5ia: *S. pombe* and *S. cerevisiae* may share some aspects of invasive transcriptional control

The *S. cerevisiae* screen manipulated strains in two ways, firstly via gene disruption and secondly via gene over-expression. This is important to bear in mind when drawing conclusions from the invasive outcome. Out of the 11 classified as non-invasive from the *S. pombe* screen that had orthologues identified as non-invasive in the *S. cerevisiae* screen, seven had nuclear roles, such as transcription factors (*Sp.adn2* and *adn3*),

chromatin silencing (*Sp.swd2*) mRNA stability (*Sp.SPAC664.03*) as well as a karyopherin (*Sp.imp1*). 6 of the *S. cerevisia*e non-invasive phenotypes were a result of gene disruptions (*spac4g8.03c,adn2, adn3, spac.6g914, hsr1* and *imp1*) therefore they probably function as positive regulators of the invasive response. The *Swd2* orthologue exhibited a non-invasive phenotype when over-expressed. This is in contrast to the *S. pombe* result, where a poorly-invasive phenotype is seen when the gene is deleted. Swd2 is a chromatin remodelling protein that is involved in histone methylation, resulting in tight wrapping of the chromatin to the nucleosomes. This makes it harder for the transcriptional machinery to access the promoters resulting in gene silencing.
14 of the *S. pombe* non- or poorly invasive strains that have orthologues in the *S. cerevisiae* screen have nuclear based roles. Seven of these orthologues exhibited invasive efficiency defects in the *S. cerevisiae* screen (discussed above), therefore seven *S. cerevisiae* orthologues did not exhibit phenotypes in the screen.
The non-invasive phenotype of the six conserved nuclear based proteins that arises from gene deletion suggests conserved methods of transcriptional control are shared between the two yeasts. However, the opposing phenotype of *swd2Δ* , and the lack of invasive efficiency aberrations for the remaining seven orthologues suggests there are also significant variations.

3.3.5ib: Invasive growth of *S. pombe* and *S. cerevisiae* utilises the cAMP signalling cascade

Two of the 11 orthologues (*sp*git3 and *sp*gpa2) that exhibited invasive defects in both the *S. pombe* and *S. cerevisiae* screen represent proteins that are involved in the cAMP signalling cascade. This signalling cascade is involved in invasion of both yeasts and represents a conserved signalling process shared between them (Gimeno et al. 1992; Amoah-Buahin et al. 2005).
Efc25 is a GEF involved in microtubule organization (Tratner et al. 1997). The *S. cerevisiae* orthologue CDC25, is linked to the cAMP signalling cascade (van Aelst et al. 1991), though this role is not seen in *S. pombe*. In *S. pombe* Efc25 is a GEF in the Scd1, Cdc42 signalling cascade which is involved in microtubule organisation (Tratner et al. 1997). The exhibition of poorly-invasive phenotype in the *S. pombe* screen suggests *Sp.*Efc25 may have a conserved role in cAMP signalling during the invasive process.

The conserved phenotype displayed by *Sp.git3Δ/SC.GPR1Δ* and *Sp.gpa2Δ/Sc.GPA2Δ* correlates to a well characterised conserved signalling cascade. As *Sp.Met14ΔSc.MET14Δ* also displays a conserved phenotype Met14 may represent a un-described conserved mechanism.

3.3.5ic: Met14 may represent a conserved mode of regulation between the two yeasts

Sp.met14 is an adenylsulphate kinase (http://old.genedb.org/genedb/pombe/). Its orthologue also exhibited a phenotype in the *S. cerevisiae* screen. However, it was capable of invasion, but its colony morphology was altered. The colony morphologies described by Fink *et al*. are a result of the growth phase called pseudohyphae, which is filament formation on the surface of the agar. *S. pombe* is not known to grow in this manner. In the *S. cerevisiae* screen *ScMET14* was over-expressed from a plasmid resulting in increased protein levels, which is the opposite effect to gene deletion. The colony morphology of *Sc.MET14* over-expression was extremely exaggerated; suggesting *Sc.*MET14 positively regulates this process. In the *S. cerevisiae* screen no effect on invasive efficiency resulting from *Sc.MET14* over-expression was noted but the extremely exaggerated colony morphology phenotype presented suggests *Sc.MET14* over expression could also causes hyper-invasion, which would correlate with the *S. pombe* non-invasive phenotype displayed by the *Sp.met14Δ.* The proposed correlation of *Sp.Met14/Sc.MET14* phenotype implies conserved role for Met14 during invasion of these two yeasts, however it is not immediately clear why sulphate assimilation would be involved during invasive growth. There are 4 other genes within the *S. pombe* genome annotated with the sulphate assimilation GO term and none of these were identified in the *S. pombe* screen suggesting a role independent of sulphate assimilation during invasion.

This concludes the comparison of *S.cerevisiae* screen data, which implies there are conserved and divergent regulation processes during filament formation. A previous screen for *S. pombe* invasion defective strains (Dodgson et al. 2009) allows further comparative analysis.

3.3.5ii: Comparative analysis of YES and LNB invasive deficiency screens

A screen of the version β Bioneer *S. pombe* deletion library for invasive defects on rich (YES) media, classified 12 strains as non-invasive (Dodgson et al. 2009) (table 3.11). The screen presented within this thesis utilised a low nitrogen base (LNB) media with an aim to identify genes required in the response to low nitrogen. The previous *S. pombe* screen utilised YES media for invasion, therefore low nitrogen would not have been the invasion-inducing factor. Comparative analysis of non-invasive strains identified between the two *S. pombe* screens may highlight processes required for induction of invasion by low nitrogen.

3.3.5iia: Strains that are non-invasive on LNB but invasive on YES

15 strains were unable to invade on LNB but able to invade on YES (table 3.11). These represent potential nitrogen sensing proteins. Nine of the 15 strains displaying this phenotype have transcriptional or chromatin remodelling roles. This suggests transcriptional regulation is a prevalent method of invasive induction in response to nitrogen deprivation.

Three strains (*gpa2Δ, git3Δ* and *git5Δ*) displaying this phenotype represent proteins with well established roles in the cAMP signalling cascade (Hoffman et al. 1991). This cascade is activated by high environmental glucose, which is present in both media types. This suggests induction of invasion by low nitrogen is more dependent on a cAMP signal.

Pyp1 is an inhibitory phosphatase for Sty1, the final kinase in the stress-activated MAPK (Shiozaki et al. 1995). There is more Sty1 activity in the *pyp1Δ* cells. As this shows decreased invasion, it suggests the stress-activated MAPK signalling cascade is a negative regulator of the invasive process on LNB but not YES.

The remaining strain displaying this comparative phenotype is *met14Δ.* Met14 is involved in methionine biosynthesis and required for sulphate assimilation. The orthologue in *S. cerevisiae* is also invasion-defective (see above). This conserved role in invasion appears to be specific for invasion induced by low nitrogen. Sulphate assimilation is linked to nitrate assimilation in plants (Koprivova et al. 2000). *S. pombe* cannot assimilate nitrate from the environment, however, sulphate assimilation during invasion may be linked to the uptake of alternative nitrogen sources such as ammonia. The nitrogen source in LNB is ammonium sulphate. Therefore ammonia uptake may be

Gene ID	Phenotype on YES	Phenotype on LNB	Gene Name	Gene Description
SPAC2F7.08c	Non-invasive	Non-invasive	*snf5*	SWI/SNF complex subunit, involved in chromatin remodelling(predicted)
SPBC21.05c	Non-invasive	Good invasion	*ral2*	Kelch repeat protein, involved in regulation of Ras1p
SPBC11B10.07c	Non-invasive	Good invasion	*ivn1*	CDC50 domain protein, implicated in signal transduction
SPAC3H8.10	Non-invasive	Good invasion	*Spo20Δ*	CRAL/TRIO domain, sec14 cytosolic factor family
SPBC30B4.03c	Non-invasive	Poorly-invasive	*adn1*	Adhesion defective protein, predicted transcriptional regulator
SPAC23D3.09	Non-invasive	Good invasion	*arp42*	SWI/SNF and RSC complex subunit, involved in chromatin remodelling
SPBC19C7.02	Non-invasive	Good invasion	*Ubr1*	Ubiquitin-protein ligase (E3)
SPAC823.05c	Non-invasive	Good invasion	*Tlg2*	SNARE protein, involved in intracellular transport
SPCC126.04c	Non-invasive	Good invasion	*Sgf73*	SAGA complex subunit, involved in chromatin remodelling
SPBC1289.10c	Non-invasive	Non-invasive	*adn2*	Transcriptional regulator, positively regulates cell-substrate adhesion
SPCC1494.10	Non-invasive	Non-invasive	*adn3*	Transcriptional regulator, positively regulates cell-substrate adhesion
SPCC895.05	Non-invasive	Poorly-invasive	*for3*	Formin, involve in actin filament bundle assembly
SPBC336.03	Invasive	Non-invasive	*efc25*	GEF for Ras1-Scd1 mating MAPK pathway
SPBC30B4.04c	Invasive	Non-invasive	*sol1*	SWI/SNF complex subunit, involved in chromatin remodelling
SPAC23H3.13c	Invasive	Non-invasive	*gpa2*	G-protein alpha subunit for glucose driven cAMP signalling
SPBC609.05	Invasive	Non-invasive	*pob3*	FACT complex component (predicted)

Table 3.11

Gene ID	Phenotype on YES	Phenotype on LNB	Gene Name	Gene Description
SPBC21C3.02c	Invasive	Non-invasive	*dep1*	Histone deacetylation, chromatin remodelling
SPCC1753.02c	Invasive	Non-invasive	*git3*	G protein coupled receptor for glucose activated cAMP signal transduction cascade
SPBC530.08	Invasive	Poorly-invasive		Transcriptional regulator (predicted)
SPBC18H10.06c	Invasive	Poorly-invasive	*swd2*	SET1 complex (TAP), involved in chromatin silencing
SPAC32A11.03c	Invasive	Poorly-invasive	*phx1*	Transcription regulator
SPAC1782.11	Invasive	Poorly-invasive	*met14*	Adenylylsulfate kinase (predicted), involved in sulphate assimilation
SPCC1223.13	Invasive	Poorly-invasive	*cbf12*	DNA binding protein
SPBC1604.08c	Invasive	Poorly-invasive	*imp1*	Karyopherin (predicted)
SPAC23E2.01	Invasive	Poorly-invasive	*fep1*	Iron-sensing transcription regulator
SPBC32H8.07	Invasive	Poorly-invasive	*git5*	G-protein beta subunit for glucose driven cAMP increase
SPAC26F1.10c	Invasive	Poorly-invasive	*pyp1*	Phosphoprotein phosphatase

Table 3.11 Comparison of invasive phenotype on YES and LNB. All gene descriptions are taken from Gene DB (http://old.genedb.org/genedb/pombe/ last accessed 11/07/2011)

coupled to sulphate assimilation. In *Met14*Δ cells this sulphate/ammonium assimilation would not be taking place, therefore the cell would not be aware of the low nitrogen levels in the environment and therefore not be induced to invade. Up-regulation of nitrogen and sulphate assimilation genes has been shown to affect the morphology of *S. cerevisiae* (Cavalieri et al. 2000), which further implicates the assimilation of these two compounds in control of morphology. This suggests a role for Met14 as a nitrogen sensor during low-nitrogen induced invasion.

The analysis of strains that represent the nitrogen induced invasive response implicate positive regulation by cAMP and negative regulation by the stress-activated MAPK pathway. These may transduce the signal to alter transcription of target set of genes that are responsible for invasion.

The strains classified as non-invasive over both screens represent the conserved processes of invasion between the two different media.

3.3.5iib: Strains that are non-invasive on LNB and YES

Five strains that were classified as non-invasive on YES also failed to invade on LNB. These strains represent the conserved invasive regulators on LNB and on YES. Adn1, Adn2 and Adn3 are transcription factors and Snf5 is part of the SWI/SNF chromatin remodelling complex (http://old.genedb.org/genedb/pombe/). This implies that some regulation at the transcriptional level is conserved. The non-invasive phenotype of *for3Δ* on YES and LNB implicates it as the crucial formin in control of invasive growth.

The final comparative phenotypic group constitutes the strains that were unable to invade on YES but able to invade on LNB. These represent the YES specific invasive machinery.

3.3.5iic: Strains that were able to invade on LNB but unable to invade on YES

Seven mutant strains, *ral2 Δ, ivn1 Δ, arp42 Δ, Sgf73Δ, Ubr1Δ, Tlg2Δ* and *Spo20Δ*, were non-invasive on YES but invasive on LNB. Tlg2 is a SNARE protein involved in vesicle transport (http://old.genedb.org/genedb/pombe/). SNARE proteins are responsible for the fusion of many membrane bound organelles within the cytoplasm and play a role in exocytosis (Carpp et al. 2007). Sgf73 is a subunit of the SAGA complex and is involved in chromatin silencing. Ubr1 is a ubiquitin E3 ligase that is responsible for the final stage of the process that targets proteins for proteosomal

degradation (http://old.genedb.org/genedb/pombe/). Spo20 is an essential protein involved in transport of secretary proteins, the *spo20* deletion in version β Bioneer *S. pombe* deletion library is only a partial deletion (Dodgson et al. 2009). Ivn1 is an uncharacterised protein that has been implicated in signal transduction. Ral2 is a GEF in the Ras1-Scd1 signalling cascade (http://old.genedb.org/genedb/pombe/).

The analysis of these strains does not highlight a process which is specific to YES invasion, but suggests there are differences in the signalling cascades that trigger the invasion on the alternate media sources.

The final comparison will be numerical, with an aim to determine any differences in complexity between the invasive processes on different media.

3.3.5iid: Quantitative comparison of YES and LNB screen

11 strains were classified in the YES screen as non-invasive. The same experimental techniques would have identified 20 in the LNB screen (table 3.11). The YES screen exclusively utilised the Bioneer *S. pombe* version β deletion library. There are nine version β strains classified as non-invasive in the LNB screen, and an additional 11 version β strains that were described as poorly invasive. As more strains were classified as non-invasive on LNB than on YES it could suggest that the invasive process is more complex on LNB than on YES.

The comparison of the YES and LNB screen has suggested a number of differences and similarities between invasion on the two media. There are conserved and divergent transcriptional control mechanisms. cAMP activation and stress-activated MAPK inhibition appear to be more prominent for LNB induced invasive growth. This comparison was facilitated by reviewing research by other groups.

3.4: Conclusions drawn from the *S. pombe* screen for invasive, morphological mutants

The screen of the *S. pombe* Bioneer deletion libraries for invasive and/or morphologically defective strains has allowed the description of a tertiary stage in the invasive process, filament formation. Analysis of the 177 represented genes suggests Fta5, a cell surface adhesin, is crucial for invasion and that its presence at the cell surface may be regulated by transcriptionally mediated cell wall remodelling. The tertiary stage of invasion, filament formation, may be the only stage where monopolar growth is required (see above) and this stage is dependent on two processes; cellular

elongation and growth site determination. Growth site determination may be regulated by a mixture of events including microtubule facilitated growth component delivery, pyridoxine level, and Cdc42 mediated signalling. Cellular elongation may arise from a down regulation of stress-activated MAPK and Rho1 signalling that results in mitotic delay.

The identification of non-invasive and poorly-invasive strains provide a model system in which to research the potential signalling pathways involved in invasion. Chapter 4 addresses the regulation of invasion via addition of exogenous compounds and analysis of alteration of growth pattern.

Chapter 4: Signal transduction during invasion

4.1: Signalling pathways for invasion

The work presented in chapter 3 described progress towards understanding the changes in morphology that occur during invasive growth. However, the signalling events that result in this transition of growth form are still poorly understood. The identification of 34 non- and poorly-invasive strains (chapter 3) provides a novel means to investigate the signal transduction that results in invasion. These strains lack proteins that may be involved in transducing the invasive signal. Therefore, supplementing their growth media with potential signalling compounds will help determine what, if any, role the proteins play within the specified signalling pathway. If the aberrant invasive phenotype is rescued by growth on the supplemented media then the represented protein may function upstream of the signalling compound. However, if the defect is not rescued, it may function downstream or in an alternative signalling pathway.

Invasion has been linked to a second messenger cAMP and a signalling phosphatase calcineurin. Low-nitrogen-induced invasion requires the cAMP signalling cascade (Amoah-Buahin et al. 2005) as shown by deletion of core components such as Git3, the G protein coupled receptor (Welton et al. 2000). Deletion of *spcc830.06*, the calcineurin b regulatory subunit results in hyper-invasive growth (chapter 3). Additionally Prevorovsky *et al.* 2009 found that growth on media containing high environmental iron induces invasion (Prevorovsky et al. 2009).

To investigate the relationship between the 34 proteins represented by the non- and poorly invasive strains and these compounds, each strain was grown on media supplemented with $FeCl_2$, 8-Br-cAMP and FK506. $FeCl_2$ creates a high iron environment, 8-Br-cAMP is a cAMP analogue and FK506 is a calcineurin inhibitor.

4.2: Results

4.2.1: The effect of 8-Br-cAMP on non-invasive and poorly invasive strains

The glucose activated cAMP signalling cascade is required for low-nitrogen induced invasion (Amoah-Buahin et al. 2005). To investigate the relationship between the proteins represented by the non- and poorly-invasive strains and the cAMP signalling cascade, each strain was grown 1cm away from a filter disc soaked in 0.1M 8-Br-cAMP (Materials and Methods). Strains were grown and washed followed by inspection of the media for invasive foci. cAMP has been implicated in regulation of filament

morphology (Prevorovsky et al. 2009) therefore the invasive strains were also subjected to microscopic observation for morphological changes.

4.2.1i: 8-Br-cAMP rescued the aberrant invasive phenotype of eight strains

Eight strains exhibited an increase in invasive efficiency when grown with 0.1M 8-Br-cAMP (table 4.1, figure 4.1). These may represent proteins required to increase intracellular cAMP during invasion. Three of these proteins have well defined roles in cAMP signalling: Git3, the glucose binding receptor, Gpa2, the G protein α subunit, and Git5, the G protein β subunit (Welton et al. 2000). *Paf1Δ, pob3Δ, imp1Δ, fep1Δ* and *Spbb7e8.02Δ* represent proteins that do not have such obvious roles upstream of an intracellular cAMP increase. Paf1 is an RNA polymerase II associated protein that aids with transcription of a subset of genes, including those in cell cycle regulation. Spbpb7E8.02 is a conserved hypothetical phospho-protein (http://old.genedb.org/genedb/pombe/). Pob3 is involved in gene silencing (Lejeune et al. 2007) and Imp1 is a karyopherin (Umeda et al. 2005). Fep1 is an iron-sensing transcriptional regulator (Pelletier et al. 2002).

4.2.1ii: 8-Br-cAMP did not rescue the aberrant invasive phenotype of 26 strains

Growth next to a filter disc soaked in 8-Br-cAMP did not affect invasive efficiency of 26 strains (figure 4.1, table 4.1). These strains represent proteins that may be targets of the cAMP signal. Alternatively, these proteins may function in different mechanisms that regulate invasion. Convergence of the cAMP signalling cascade with another signalling pathway(/s) may be essential to elicit an invasive response. 11 of these strains (*snf5Δ, sol1Δ, dep1Δ, adn1Δ, adn2Δ, adn3Δ, hsr1Δ, spbc530.08Δ, swd2Δ, phx1Δ* and *cbf12Δ*) have nuclear-based roles such as transcription factors (http://old.genedb.org/genedb/pombe/). Two strains (*ccr1Δ* and *ogm1Δ*) represent integral membrane proteins that are involved in ergosterol biosynthesis and cell wall remodelling respectively (Willer et al. 2005). *Spac6g9.14Δ* and *spac4g8.03cΔ* represent RNA binding proteins and *spbc16g5.02Δ* represents a ribokinase (http://old.genedb.org/genedb/pombe/). *Atg8Δ* and *atg14Δ* represent autophagy associated proteins and *vps45Δ* represents a protein involved in vesicle transport (http://old.genedb.org/genedb/pombe/). Finally, *pyp1Δ* represents a protein phosphatase

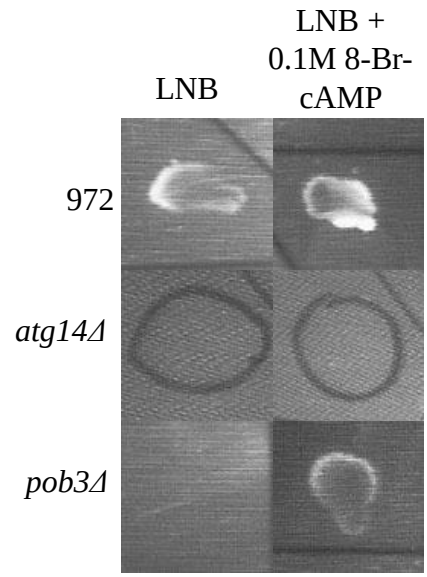


Figure 4.1 Effect of 0.1M 8-Br-cAMP on efficiency of invasion of non-invasive and poorly-invasive strains. Some strains exhibited a rescue of aberrant invasive phenotype (represented by *pob3Δ)* whilst others do not (represented by *atg14Δ).*

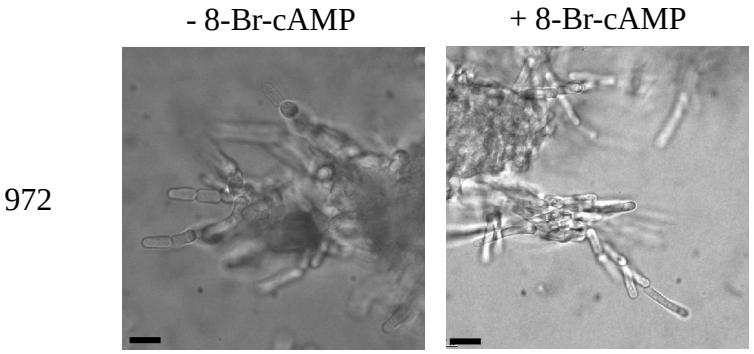


Figure 4.2 Effect of 8-Br-camp on filament formation of *S. pombe* 972. Filaments exhibit an elongated morphology in the presence of 8-Br-cAMP. Bar 10μM.

Gene ID	Phenotype	Gene Name	Gene Description	1	2	3	4
SPBC336.03	Non-invasive	*efc25*	GEF for Ras1-Scd1 pathway,	No	No	No	No
SPAC2F7.08c	Non-invasive	*snf5*	chromatin remodeling complex	No	No	Yes	No
SPBC30B4.04c	Non-invasive	*sol1*	transcriptional regulator (predicted)	No	No	Yes	No
SPBC609.05	Non-invasive	*pob3*	FACT complex component	No	Yes	Yes	Yes
SPBC21C3.02c	Non-invasive	*dep1*	Histone deacetylation,	No	No	Yes	No
SPBC1289.10c	Non-invasive	*adn2*	transcriptional regulator (predicted)	No	No	Yes	No
SPCC1494.10	Non-invasive	*adn3*	transcriptional regulator (predicted).	No	No	Yes	No
SPCC1753.02c	Non-invasive	*git3*	G-protein coupled receptor, required for	Yes	Yes	Yes	Yes
SPAC22A12.07c	Non-invasive	*ogm1*	protein O-mannosyltransferase	No	No	No	Yes
SPAC4G8.03c	Non-invasive		RNA-binding protein	No	No	Yes	No
SPAC2G11.03c	Non-invasive	*vps45*	vacuolar sorting protein	No	No	Yes	Yes
SPAC8E11.05c	Non-invasive		conserved fungal protein	No	No	No	No
SPAC3H1.11	Non-invasive	*hsr1*	transcription factor	No	No	Yes	No
SPAC1F8.06	Non-invasive	*fta5*	Cell surface glycoprotein	No	No	Yes	No
SPBC18H10.19	Non-invasive	*atg14*	human UVRAG (UV radiation resistance)	No	No	Yes	Yes
SPBC29A10.01	Non-invasive	*ccr1*	NADPH-cytochrome p450 reductase	No	No	No	Yes
SPAC18G6.13	Non-invasive		sequence orphan	No	No	Yes	No
SPBC16G5.02c	Non-invasive		ribokinase	No	No	Yes	No
SPBC530.08	Poorly-invasive		transcriptional regulator (predicted)	No	No	Yes	No
SPBC30B4.03c	Poorly-invasive	*adn1*	adhesion defective protein, predicted	No	No	Yes	Yes
SPBC18H10.06c	Poorly-invasive	*swd2*	SET1 complex (TAP), involved in	No	No	Yes	No
SPAC23H3.13c	Non-invasive	*gpa2*	G-protein alpha subunit for glucose	Yes	Yes	Yes	Yes
SPAC32A11.03c	Poorly-invasive	*phx1*	transcription regulator	No	No	Yes	Yes

Table 4.1

Gene ID	Phenotype	Gene Name	Gene Description	1	2	3	4
SPAC1782.11	Poorly-invasive	*met14*	adenylylsulfate kinase	Yes	No	Yes	Yes
SPCC895.05	Poorly-invasive	*for3*	Formin, involved in actin cable assembly	No	No	Yes	Yes
SPCC1223.13	Poorly-invasive	*cbf12*	DNA binding protein (inferred from	No	No	Yes	Yes
SPBC1604.08c	Poorly-invasive	*imp1*	karyopherin (predicted).	No	Yes	Yes	Yes
SPAC23E2.01	Poorly-invasive	*fep1*	iron-sensing transcription	No	Yes	Yes	No
SPBC32H8.07	Poorly-invasive	*Git5*	G-protein beta subunit for glucose	Yes	Yes	Yes	Yes
SPAC26F1.10c	Poorly-invasive	*pyp1*	phosphoprotein phosphatase, de-	No	No	Yes	No
SPBPB7E8.02	Poorly-invasive		conserved protein (fungal bacterial	Yes	Yes	Yes	Yes
SPAC6G9.14	Poorly-invasive		RNA-binding protein	Yes	No	Yes	Yes
SPBC15D4.07c	Poorly-invasive	*atg8*	autophagy associated protein Atg8	No	No	No	No
SPAC664.03	Poorly-invasive		RNA polymerase II associated Paf1	Yes	Yes	Yes	Yes

Table 4.1 Effect of signalling compounds on invasive efficiency of non-invasive and poorly invasive strains. 1: Does 8-Br-cAMP elongate filaments 2: Does 8-Br-cAMP rescue aberrant invasive phenotype 3: Does $FeCl_2$ rescue aberrant invasive phenotype 4: Does FK506 rescue aberrant invasive phenotype. Gene descriptions reference; http://old.genedb.org/genedb/pombe/

that targets Sty1 MAPK (Dal Santo et al. 1996), *fta5Δ* represents a cell surface glycoprotein (Linder et al. 2008) and *met14Δ* represents a protein involved in methionine biosynthesis and sulphate assimilation (http://old.genedb.org/genedb/pombe/).

This further characterises the effects of 8-Br-cAMP on invasive efficiency of the non-invasive and poorly invasive strains, but could microscopic observation reveal anything more about the role of cAMP during invasion?

4.2.1iii: 8-Br-cAMP affected filament morphology

Microscopic observation of *S. pombe* 972 invasive foci grown next to the 8-Br-cAMP soaked filter disc, revealed elongated cells within the filaments (figure 4.2). This effect on morphology has previously been reported (Prevorovsky et al. 2009).

The ability of 8-Br-cAMP to affect filament morphology as well as invasive efficiency raises a question: are these effects separable? This was investigated by microscopic observation of strains whose aberrant invasive phenotype were and were not rescued by growth next to a 0.1M 8-Br-cAMP soaked filter disc.

4.2.1iv: 8-Br-cAMP rescued the aberrant invasive efficiency phenotype without affecting filament morphology

Growth next to a 0.1M 8-Br-cAMP soaked filter disc rescued the invasive efficiency defect but did not alter the filament morphology of three strains, *pob3Δ, imp1Δ* and *fep1Δ* (figure 4.3). This implies the effect of 8-Br-cAMP on invasion and filament formation is separable and there is an invasion-associated as well as a filament morphology-associated cAMP signal. Additionally, it implies that Pob3, Imp1 and Fep1 are involved in both invasion-associated and filament morphology-associated cAMP signalling. They may function upstream of cAMP during invasion (therefore 8-Br-cAMP rescues aberrant invasive phenotype), but downstream of cAMP during filament formation (therefore no elongation of filament morphology). Pob3, Imp1 and Fep1 all localise to the nucleus (http://old.genedb.org/genedb/pombe/).

Two strains (*met14Δ* and *spac6g9.14Δ*) exhibited elongated filament morphology but no rescue of invasive efficiency defect (figure 4.3, table 4.1). Met14 and Spac6g9.14 are cytoplasmic proteins (http://old.genedb.org/genedb/pombe/).

The exhibition of separable phenotypes suggests cAMP has separate functions for invasion and for development of filament morphology. Microscopic observation of the

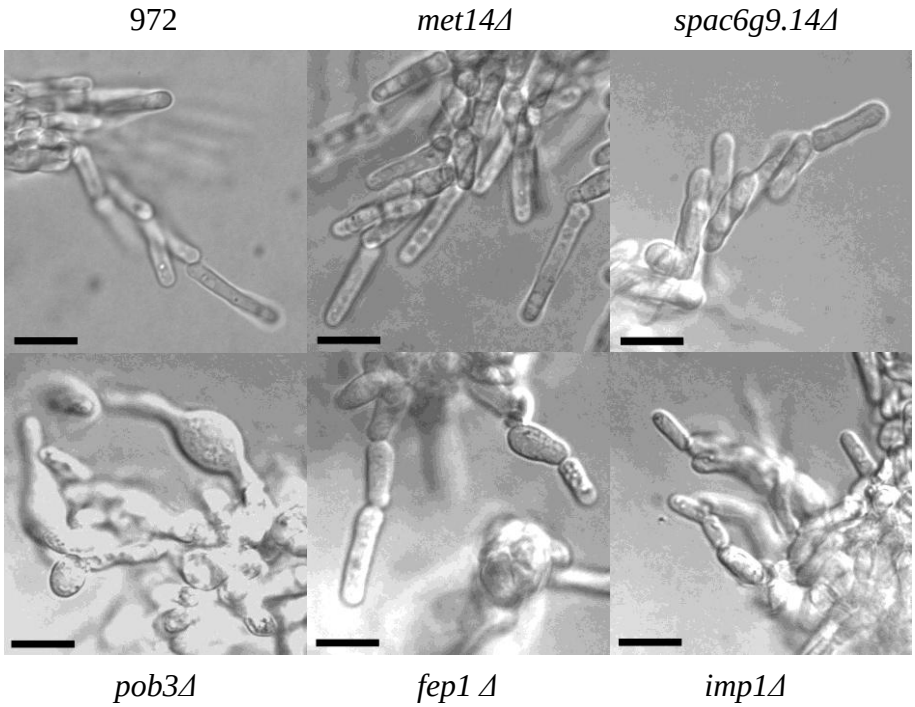


Figure 4.3 Effect of 8-Br-cAMP on filament morphology of *met14Δ spac6g9.14Δ, pob3Δ, fep1Δ* and *imp1Δ*. 8-Br-cAMP elongates filaments of *met14Δ* and *spac6g9.14Δ* but does not elongate filaments of *pob3Δ, fep1Δ* and *imp1Δ*. Bar 10μM.

poorly-invasive strains when grown next to 0.1M 8-Br-cAMP filter disc may reveal which of the represented proteins function in filament morphology-associated signalling as well as invasion-associated cAMP signalling. As the non-invasive or poorly-invasive strains did not exhibit aberrations in filament formation the elongation of their filaments by 8-Br-cAMP does not suggest they function upstream of cAMP during filament formation. However, strains that do not exhibit elongated filaments on 8-Br-cAMP may represent proteins that function downstream of cAMP during the filament morphology-associated cAMP signal.

4.2.1v: Growth on 8-Br-cAMP does not alter filament morphology of eight strains

Eight of the poorly-invasive strains (*spbc530.08Δ, adn1Δ, swd2Δ, phx1Δ, cbf12Δ atg8Δ for3Δ* and *pyp1Δ)* did not exhibit elongated filaments when grown next to a 0.1M 8-Br-cAMP soaked filter disc (table 4.1). As *S. pombe* 972 exhibits an elongated filament morphology in the presence of 8-Br-cAMP, this suggests the represented proteins may function downstream of the filament morphology-associated cAMP signal. Five of these (*spbc530.08Δ, adn1Δ, swd2Δ, phx1Δ* and *cbf12Δ*) are nuclear proteins such as transcriptional regulators and chromatin remodelling factors (http://old.genedb.org/genedb/pombe/). For3 is responsible for assembly of actin cables at sites of cell growth (Feierbach et al. 2001). Pyp1 is a protein phosphatase that functions in the stress-activated MAPK cascade (Dal Santo et al. 1996; Mukaiyama et al. 2010)

4.2.1vi: Conclusion from 8-Br-cAMP supplementation experiments

These results have confirmed that glucose activated cAMP signalling is involved in invasion. Additionally, they suggest that cAMP functions during filament formation and the effects of cAMP on these two stages of the invasive process are separable.

Three strains (*pob3Δ, imp1Δ* and *fep1Δ*) have been implicated in regulation of both cAMP signal transduction events during the invasive process. To investigate whether or not this is an invasion-specific role, the effect of these proteins on a well documented cAMP target was studied.

4.2.2: Imp1, Fep1 and Pob3 do not alter cAMP mediated regulation of *fbp1*

Fbp1 is a well-documented transcriptional target of the glucose activated cAMP signal transduction cascade (Vassarotti et al. 1985). Fbp1 has previously been fused to

lacZ to form a reporter system for this cascade (Hoffman et al. 1990). This allows investigation of the relationship between the invasion and filament morphology-associated cAMP signal and a known downstream target of cAMP signalling. To do this, strains that represent proteins that may be involved in both invasion and filament morphology-associated cAMP signalling (*pob3Δ, imp1Δ* and *fep1Δ*) and two controls (*git3Δ* and *spac664.03Δ*) were mated to the Fbp1 reporter strain and the appropriate progeny were selected. The *fep1Δ Fbp1:lacZ*, *imp1Δ Fbp1:lacZ* and *pob3Δ Fbp1:lacZ* reporter strains were then subjected to surface colony and invasive X-gal assays (Materials and Methods) to determine whether or not the deleted genes effected *fbp1* expression.

None of the deletion strain/reporter hybrids exhibited altered Fbp1 expression during yeast phase or invasive growth (figure 4.4). However, the control (*git3Δ*) which should have exhibited higher Fbp1 expression did not appear to, therefore these results may not be reliable. The yeast phase assay is not directly comparable to the invasive assay as different methods were used.

4.2.3: The affect of 4mM $FeCl_2$ on the non-invasive and poorly invasive strains

Iron has been shown to be a potent stimulant of invasion (Prevorovsky et al. 2009). To investigate the relationship between the non- and poorly-invasive strains identified in the screen and iron, each strain was grown on 4mM $FeCl_2$ media. Strains were grown and washed followed by inspection of the media for invasive growth. As iron has not been implicated in filament formation the morphology of the strains on 4mM $FeCl_2$ was not inspected.

4.2.3i: 4mM $FeCl_2$ rescued the aberrant invasive phenotype of 29 strains

Growth on 4mM $FeCl_2$ LNB media rescued the invasive defect of 29 strains (figure 4.5, table 4.1). The strains rescued included all the cAMP signalling associated components (*git3Δ, gpa2Δ* and *git5Δ*) and all the transcription factors.

4.2.3ii: 4mM $FeCl_2$ did not rescue the aberrant invasive phenotype of five strains

Five strains (*efc25Δ, ogm1Δ, spac8E11.05Δ, ccr1Δ* and *atg8Δ*) did not exhibit increased invasive efficiency on 4mM $FeCl_2$. Efc25 is a GEF for Ras1 family protein (Tratner et

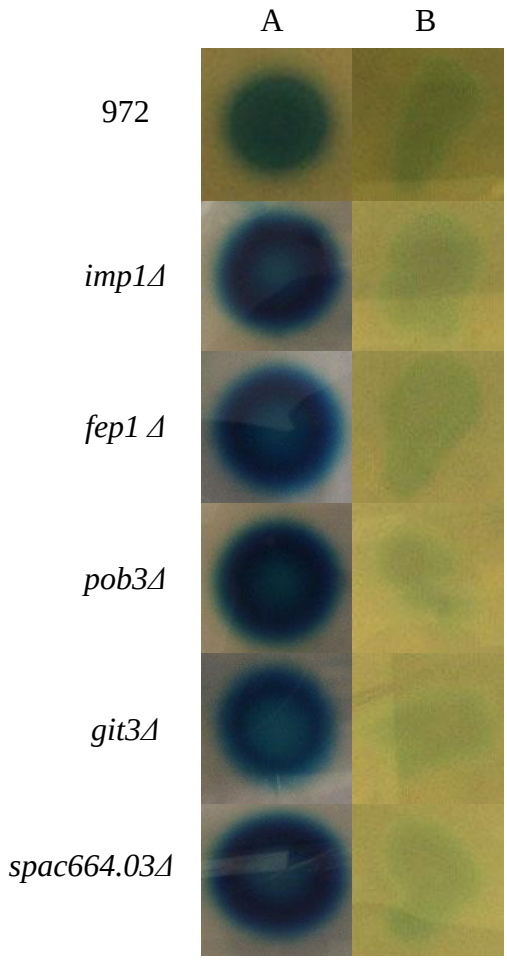


Figure 4.4. The effect of. *imp1Δ*, *pob3,Δ* *fep1Δ* ,*git3Δ* and *spac664.03 Δ* on Fbp1-lacZ expression. *Git3Δ* and *Spac664.03Δ* were used as controls. Hybrid progeny were subjected to a yeast-phase (A) and invasive (B) Xgal assays. The two assays are not directly comparable as different x gal assay methods have been used.

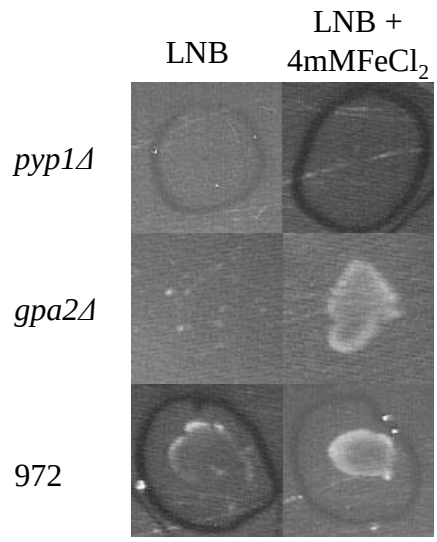


Figure 4.5 Effect of 4mM $FeCl_2$ on invasive growth of non- and poorly-invasive strains. Iron rescues aberrant invasive phenotype of 29 strains (represented by *gpa2Δ)* but does not rescue defects of five strains (represented by *pyp1Δ*).

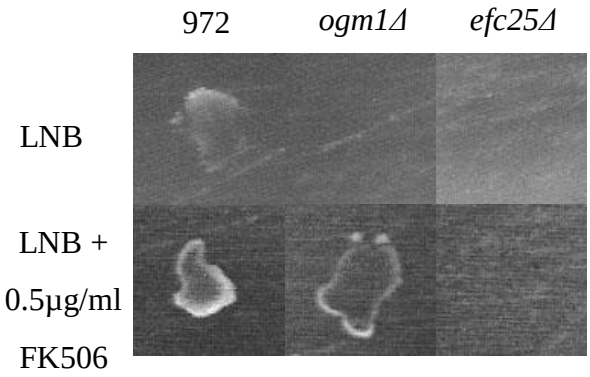


Figure 4.6 Effect of 0.5µ/ml FK506 on invasive growth of non-invasive and poorly-invasive strains. FK506 rescues the aberrant invasive phenotype of 17 strains (represented by *ogm1Δ)* but not of a different 17 strains (represented by *efc25Δ*).

al. 1997). Ccr1 is proposed to be involved in ergosterol biosynthesis based on homology to *S. cerevisiae* NCP1 (Lin et al. 2009). Ogm1 is an endoplasmic reticulum localised o-mannoysltrasnferase (Willer et al. 2005). Spac8e11.05 is a conserved hypothetical protein that has no orthologues in a *S. cerevisiae* and Atg8 is involved in the CVT pathway and autophagy (http://old.genedb.org/genedb/pombe/).
These results suggest stimulation of invasion by excess iron induces invasion by over-riding the normal invasive response.

4.2.4: The effect of FK506 on the non-invasive and poorly invasive strains

Calcineurin is a protein phosphatase that has been implicated in control of invasion by the exhibition of a hyper-invasive phenotype by *spcc830.06Δ,* the calcineurin b regulatory subunit. To investigate the relationship between calcineurin signalling and the non-/poorly-invasive strains, each was grown on 0.5µg/ml FK506 LNB media. FK506 mimics the deletion of calcineurin b (Sio et al. 2005) therefore inhibits calcineurin signalling. Strains were grown on 0.5µg/ml FK506 LNB media and subsequently washed followed by inspection of the media for invasive growth.

4.2.4i: 0.5µg/ml FK506 rescued the aberrant invasive phenotype of 17 strains

17 strains exhibited significantly increased invasive efficiency upon growth on 0.5µg/ml FK506 LNB (table 4.1, figure 4.6). Six of these strains have nuclear based roles such transcriptional regulators (http://old.genedb.org/genedb/pombe/). Three strains (*git3Δ, gpa2Δ* and *git5Δ*) represent the glucose activated cAMP signalling component proteins (Welton et al. 2000). *Ogm1Δ* and *ccr1Δ* are involved in cell wall and cell membrane remodelling respectively (Willer et al. 2005; Lin et al. 2009) and *for3Δ* represents a formin responsible for actin assembly (Feierbach et al. 2001). The remaining strains (*met14Δ, Spac6G9.14Δ, atg14Δ* and *vps45Δ*) represent proteins involved in sulphate assimilation, RNA binding, autophagy and vacuole protein transport respectively (http://old.genedb.org/genedb/pombe/). Finally *Spbpb7E8.02Δ* represents a conserved fungal phosphoprotein (http://old.genedb.org/genedb/pombe/).

4.2.4ii: 0.5µg/ml FK506 did not rescue the aberrant invasive phenotype of 17 strains

17 strains did not exhibit an increase in invasive efficiency on 0.5µg/ml FK506 LNB (table 4.1, figure 4.6). 11 of the strains represent proteins with nuclear based roles such

as transcription factors and chromatin remodelers (http://old.genedb.org/genedb/pombe/). *Spac4g8.03cΔ* represents an RNA binding protein and *spac8e11.05cΔ* represents a conserved phosphoprotein (http://old.genedb.org/genedb/pombe/). *Efc25Δ* represents a GEF for Ras1 (Tratner et al. 1997) and *fta5Δ* represents is a cell surface glycoprotein (Linder et al. 2008). Finally *pyp1Δ* represents a phosphoprotein phosphatase that regulates the stress-activated Sty1 MAPK (Dal Santo et al. 1996) and *atg8Δ* represents an autophagy associated protein (http://old.genedb.org/genedb/pombe/).

The invasive growth displayed by 17 of the non-/poorly-invasive strains on 0.5µg/ml FK506 LNB further implicates calcineurin in the negative regulation of invasive growth.

4.3: Discussion

S. pombe is a relatively new model organism for studying invasive growth and there is still much to learn concerning the signalling processes involved in this growth mode. The non-invasive and poorly-invasive strains identified in chapter 3 provide a novel means for investigating the signalling process during invasion. The addition of 8-Br-cAMP, $FeCl_2$ and FK506 to the growth media of these strains implicated various proteins within the represented signalling processes.

The addition of these potential signalling compounds to the growth media of the non-invasive and poorly invasive strains provides a crude upstream/downstream test for the role of the represented proteins within the represented pathway (figure 4.7). If a non- or poorly-invasive strain presents an invasive phenotype on media supplemented with the signalling compound then the phenotype can be described as “rescued”. This discussion is limited to the strains that exhibit a rescued phenotype as these represent proteins that may function upstream of the specified signalling compound. The proteins that may function downstream of a compound will not highlight any mechanisms required for stimulating that signal therefore individual strains will not be considered. Additionally the failure of a compound to rescue a non- or poorly-invasive phenotype does not necessarily mean the represented protein functions downstream of that signalling compound. The protein could simply not function in the specified signalling pathway (figure 4.7), therefore supplementing the strain with the signalling compound would not rescue the aberrant invasive phenotype.

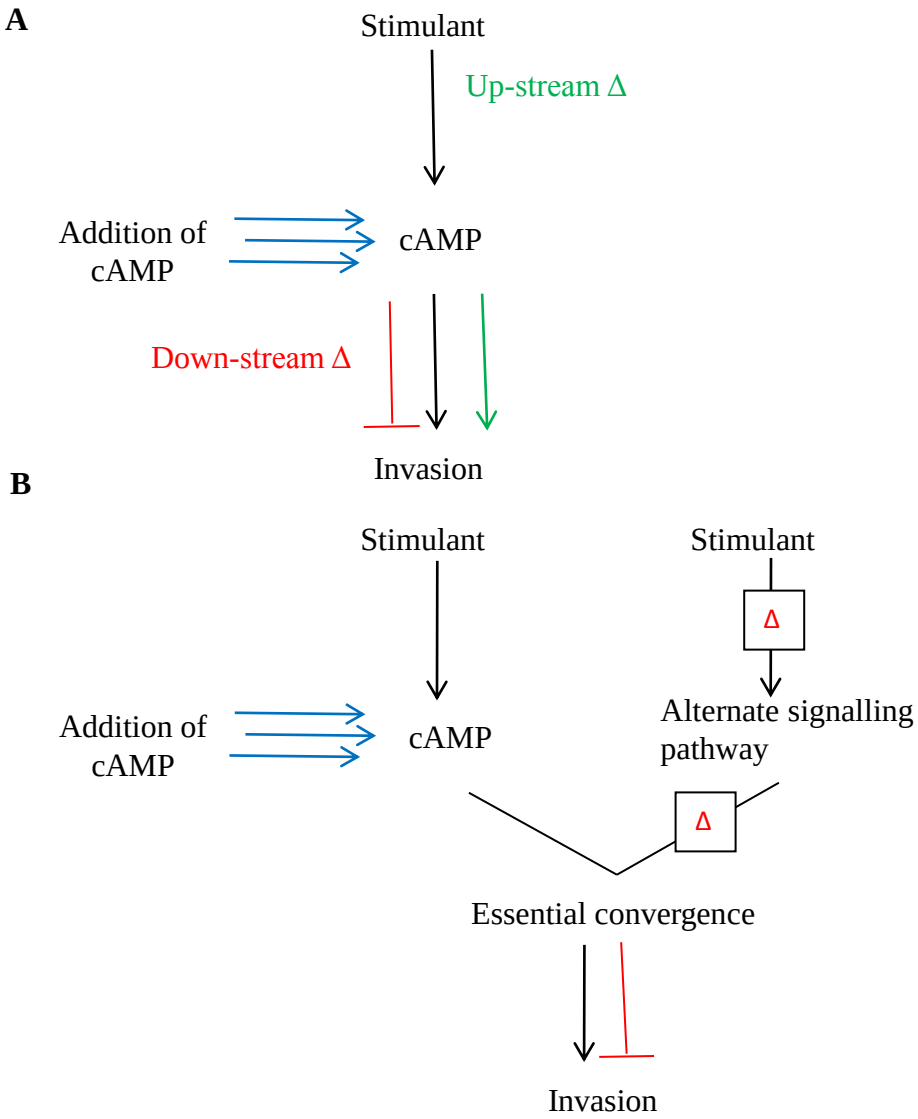


Figure 4.7 Model of rescue of aberrant invasive phenotype by addition of 8-Br-cAMP. (A) If the protein encoded by the gene deletion functions upstream of cAMP (green arrow), then addition of exogenous cAMP analogue (blue arrows) will rescue the invasive defect . If the deleted gene encodes a protein that functions downstream of the increased cAMP (red arrow) then the addition of exogenous cAMP analogue will not rescue the invasive defect. (B) The cAMP signalling cascade may have to converge with an additional signalling cascade. If the protein encoded by the gene deletion (Δ) functions in an alternate but essential signalling cascade, then addition of cAMP will not rescue the aberrant phenotype.

The first pathway to be discussed is the glucose activated cAMP signalling cascade.

4.3.1: cAMP controls two distinct events during invasive growth

Growth next to a filter disc soaked in 0.1M 8-Br-cAMP can cause an increase in invasive efficiency (Amoah-Buahin et al. 2005). Prevorovsky *et al.* proposed that cAMP drives filament formation but not invasion (Prevorovsky et al. 2009). The results presented here do not support this proposal as eight deletion strains exhibited rescued invasive efficiency when grown in the presence of 8-Br-cAMP. If cAMP signalling was not involved in invasion, then this rescue of aberrant invasive phenotype would not be seen. Additionally, the data presented here showed 8-Br-cAMP can cause elongation of cells within the filaments. Section 4.2.1.iv described the effect of 8-Br-cAMP on invasive efficiency but not filament morphology and vice versa. This suggests the effect of 8-Br-cAMP on these two phenotypes is separable.

Analysis of the strains that present an increase in invasive efficiency may identify proteins that function upstream of a cAMP increase.

4.3.1i: Nuclear factors may function to amplify cAMP signal

Invasive media contains a glucose concentration that stimulates cAMP production (Amoah-Buahin et al. 2005). Glucose stimulated cAMP production does not always result in invasive growth. As addition of excess cAMP analogue induces invasion it suggests the cAMP signal may be amplified to elicit an invasive response. Eight strains exhibited a rescue of invasive efficiency defect with 8-Br-cAMP. Three of these strains (*gpa2Δ, git3Δ* and *git5Δ*) represent well-defined components of glucose stimulated cAMP production (Welton et al. 2000). Growth with a cAMP analogue overcomes the requirement for these proteins, therefore the aberrant invasive phenotype is rescued. The five remaining strains *(paf1Δ, pob3Δ, imp1Δ, fep1Δ* and *Spbb7e8.02Δ)* that exhibited a rescue of aberrant invasive phenotype do not represent such obvious proteins in cAMP signalling. These may be involved in the amplification of cAMP signal. Pob3, Fep1, and Paf1 are all nuclear-localised proteins (http://old.genedb.org/genedb/pombe/). Fep1 is an iron-sensing transcription factor (Pelletier et al. 2002), Pob3 is involved in gene silencing (Lejeune et al. 2007) and Paf1 is required for elongation during transcription (http://old.genedb.org/genedb/pombe/). Additionally Imp1 is a karyopherin (Umeda et al. 2005). This suggests the amplification of the cAMP signal that may be required for invasion may be transcriptionally

regulated. Spbb7E08.02 is a conserved hypothetical phospho-protein (http://old.genedb.org/genedb/pombe/) that could potentially regulate the translocation of redundant factors to the nucleus via Imp1. This may then activate the nuclear proteins and cause subsequent amplification of the cAMP signal.
Alternatively the excess cAMP signal may over-ride the requirement of an additional invasive signal.

4.3.1ii: cAMP analogue may over-ride requirement for another invasion associated signal

Invasion may result from a combination of cellular signalling processes. The glucose activated cAMP signal may combine with an additional signal, for example one stimulated by low nitrogen, to elicit the invasive response (figure 4.7). In this scenario if the strains *(paf1Δ, pob3Δ, imp1Δ, fep1Δ* and *Spbb7e8.02Δ)* whose aberrant invasive phenotype was rescued by 8-Br-cAMP represent proteins within this additional signalling cascade the excess cAMP analogue may override the requirement for this additional signal.
cAMP binds to the regulatory subunits of protein kinase A (PKA) which causes their release from the catalytic subunit and subsequent activation. Thevelein *et al.* (2005), suggest that in *S. cerevisiae* nitrogen can activate PKA independently of cAMP (Thevelein et al. 2005). Potentially *paf1Δ, pob3Δ, imp1Δ, fep1Δ* and *Spbb7e8.02Δ* could represent proteins that are involved in a cAMP independent activation of PKA. Perhaps both these pathways lead to an additive effect on PKA activation to elicit the invasive response. By supplying excess cAMP analogue, the cAMP pathway may compensate for the loss of the cAMP independent PKA activation.
The individual analysis of strains that exhibited a rescued aberrant invasive phenotype when grown next to a filter disc soaked in 0.1M 8-Br-cAMP may indicate possible mechanisms for the increase in cAMP signal. The number of strains that were not rescued by 8-Br-cAMP suggests the involvement of additional signalling cascades.

4.3.1iii: Additional signalling cascades are probably involved during the invasive response

The majority (24 out of 34 strains) of the non- and poorly-invasive strains were not rescued by growth next to a filter disc soaked in 0.1M 8-Br-cAMP. The specific function of the represented proteins will not be considered here (see above, 4.3:

discussion). However the number of strains whose phenotype is not rescued may be informative. The large number of strains that were not rescued suggests there is another signalling process that combines with the glucose activated cAMP signalling cascade to elicit an invasive response (figure 4.7).

This concludes analysis of the strains that exhibited a rescue of aberrant invasive efficiency when grown next to a filter disc soaked in 0.1M 8-Br-cAMP. The results suggest there is an invasion-associated cAMP signalling cascade. The interaction of this invasion-associated cAMP signalling cascade with a known downstream target of the cAMP pathway was studied by creating hybrid reporter strains.

4.3.1iv: *Fbp1* expression is not effected by Imp1, Fep1 or Pob3 under invasive and non-invasive conditions

Fbp1 is a downstream target for cAMP mediated signalling (Vassarotti et al. 1985). To see whether potential upstream invasion-associated cAMP signalling components affect this known transcriptional target, fbp1-lacZ/deletion strain hybrids were created and subjected to X-gal assays. *Imp1Δ, pob3Δ* and *fep1Δ* were chosen to create the hybrids as they may represent upstream invasion-associated cAMP signal regulators.

These assays indicate that neither Imp1, Fep1 nor Pob3 affect *fbp1* expression. This does not mean they are not involved in invasion-associated cAMP signalling but suggests the downstream targets of signalling may vary in comparison to the well documented targets of "normal" cAMP signalling such as *fbp1*. However, the control strain (*git3Δ*) did not produce the expected result, therefore this potential result requires further investigation. Git3 is the transmembrane receptor of the glucose activated cAMP signalling cascade (Welton et al. 2000). Git3 senses high external glucose levels and initiates a signal that results in *fbp1* repression. Therefore in *git3Δ* cells this signal would not be transduced and *fbp1* expression would not be repressed, therefore a stronger reporter signal is exhibited. This is not represented in figure 4.4 and suggests the methods used were not reliable. The strength of reporter expression is dependent on the number of cells within the colonies. Potentially the cell counts were not accurate enough. This could result in the *git3Δ* displaying weaker reporter strength as there are less cells than the other strains being assayed.

4.3.2: Analysis of $FeCl_2$ effect on invasion of the non-invasive and poorly invasive strains

Iron has been implicated as a stimulant of invasion (Prevorovsky et al. 2009), therefore the effect of high environmental iron on the 34 non-/poorly-invasive strains was studied via growth on 4mM $FeCl_2$ LNB media. Addition of 4mM $FeCl_2$ to the growth media creates a high Fe^{2+} concentration in the growth media.

4.3.2i: Stimulation of invasion by Fe2+ may by-pass normal invasive signalling components

Addition of 4mM $FeCl_2$ to LNB media rescued the aberrant invasive phenotype of 29 strains (table 4.1). The effect of Fe^{2+} on invasion may be a response to escape from the highly toxic environment. The high level of Fe^{2+} may stimulate the invasive response by by-passing some of the proteins required during the response to low nitrogen. As the majority of strains were rescued by the addition of Fe^{2+} to the media it suggests the intervention of the Fe^{2+} response functions quite late during the signalling pathways that results in invasion.

Growth on 4mM $FeCl_2$ LNB rescues aberrant invasive phenotypes of all strains representing the cAMP associated signalling components (*git3Δ, gpa2Δ* and *git5Δ*). Growth of all the strains representing nuclear proteins was also rescued. This suggests Fe^{2+} acts downstream of cAMP signalling and transcriptional control. In mammalian cells iron can affect the translation efficiency of specific genes (Thomson et al. 1999). Although no such regulatory mechanism has been identified in yeast, this result could suggest a similar mechanism is in place. If this is true Fe^{2+} stimulated invasion may by-pass the requirement of transcriptional regulation of invasion by directly affecting the translation of unidentified target proteins.

Additionally *pyp1Δ, met 14Δ* and *spbc16g5.02cΔ* exhibited a rescued phenotype on $FeCl_2$ but not cAMP. Pyp1 is a phosphorylase (Dal Santo et al. 1996). Met14 and Spbc16g5.02c are kinases (Met14 – adenyly sulphate kinase and Spbc16G5.02c – a ribokinase) (http://old.genedb.org/genedb/pombe/). This could suggest iron by-passes the requirement for a phospho-relay event. Three further strains (*spac18g6.13Δ* , *spac6g9.14Δ* and *spac4g8.03cΔ*) exhibited a rescued phenotype on $FeCl_2$ but not cAMP. These represent a sequence orphan and two RNA binding proteins respectively (http://old.genedb.org/genedb/pombe/). This implies there are processes involved in invasion that are not yet understood.

Strains that represent previously considered effectors of the invasive response such as *fta5Δ* and *for3Δ* can be forced to invade on 4mM $FeCl_2$. This supports the idea that stimulation of invasion by excess iron induces invasion via over-riding the normal invasive response.

4.3.2ii:The aberrant invasive phenotype of *Fep1Δ* is rescued on 4mM $FeCl_2$

Fep1 is an iron-sensing transcriptional regulator (Pelletier et al. 2002). It is responsible for down regulation of iron assimilation genes and in-direct up-regulation of iron storage and iron-associated complexes in a high iron environment. Previously Prevorovsky *et al.*(2009), found that high iron does not rescue the invasive defect of *fep1Δ*. The result presented here does not correlate with this as 4mM $FeCl_2$ rescues the aberrant invasive phenotype of *fep1Δ*. There are a number of differences between the two bodies of work. Crucially, Prevorovsky et al, 2009, used a relatively low cell density compared to the result presented here. Variation in cell density has been shown to effect invasive efficiency (unpublished data) therefore this may be the cause of the difference in *fep1Δ* phenotype on 4mM $FeCl_2$.

4.3.3 Analysis of calcineurin function during invasive growth

FK506, a calcineurin inhibitor, was added to the growth medium of the non-invasive and poorly-invasive strains. FK506 mimics the deletion of calcineurin. 17 strains exhibited a rescued aberrant invasive phenotype when grown on 0.5µg/ml FK5O6 and 17 did not (table 4.1). FK506 is an inhibitor of a cellular protein and does not represent a potential signalling compound like 8-Br-cAMP and $FeCl_2$. Therefore, the results must be interpreted in a different manner. A rescue of aberrant invasive phenotype by FK506, may suggest the represented proteins regulate calcineurin inhibition of invasion.

4.3.3i: Calcineurin is a negative regulator of the invasive response

Calcineurin deletion results in hyper-invasion, therefore calcineurin may be a negative regulator of invasive growth (chapter 3). Calcineurin may inhibit invasive growth in non-invasive conditions. Upon environmental changes that induce invasion, the inhibitory role of calcineurin may be down regulated to achieve an invasive response. The strains that exhibited a rescue of aberrant invasive phenotype on 0.5µg/ml FK506 may represent proteins that down-regulate calcineurin during invasion.

4.3.3ii: Calcineurin inhibition functions downstream of cAMP

The strains that represent the cAMP signalling cascade (*git3Δ, gpa2Δ* and *git5Δ*) all exhibited a rescue of aberrant invasive phenotype on 0.5µg/ml FK506 LNB. This suggests that cAMP signalling could result in calcineurin inhibition to elicit an invasive response.

8 strains (*gpa2Δ, git5Δ, for3Δ, spac6g9.14Δ, atg14Δ, vps45Δ, spbpb7e08.02Δ* and *met14Δ*) that exhibited a rescue of aberrant invasive phenotype on 0.5µg/ml FK506 LNB are cytoplasmic proteins and six are nuclear proteins (http://old.genedb.org/genedb/pombe/). The remaining three (*git3Δ ogm1Δ* and *ccr1Δ*) strains represent integral membrane proteins (http://old.genedb.org/genedb/pombe/). This suggests that regulation of calcineurin may be achieved cytoplasmically and transcriptionally.

4.3.3iii: Two proposed effectors of the invasive response exhibited a rescued phenotype on FK506 media

Two strains (*ogm1Δ* and *ccr1Δ*) that exhibited a rescue of aberrant invasive phenotype on 0.5µg/ml FK506 LNB but not with cAMP or $FeCl_2$ represent putative effectors of the invasive response (chapter 3). Ogm1 is thought to be involved in remodelling the cell wall Ccr1 has been implicated in mediation of the Cdc42 signal as well as membrane biosynthesis. The phenotype presented on FK506 media implicates a role in repressing calcineurin. As this repression appears to intervene downstream of cAMP these proteins may still be considered effectors of the invasive response though they may have dual functions: a) to physically alter the cell shape during invasion and b) to repress calcineurin thus facilitating invasion.

This concludes the compound specific analysis of strains that were rescued of aberrant invasive phenotype. Combined analysis of the results my provide further insights into regulation of signalling events that result in invasion.

4.3.4: Combined analysis of the increased invasive efficiency results

Comparison of the strains rescued by growth with 8-Br-cAMP and FK506 may identify relationships between the two signalling pathways.

4.3.4i: Calcineurin inhibition may function at multiple points during the invasive response

Some strains (table 4.1) that exhibited a rescue of aberrant invasive phenotype on 0.5µg/ml FK506 LNB also exhibited a rescued phenotype with 8-Br-cAMP. If the sole purpose of the represented protein is to inhibit calcineurin as suggested in figure 4.8, then by analysing whether or not cAMP also rescue the aberrant invasive phenotype of the strain it is possible to elucidate whether the calcineurin signal feeds in above or below cAMP. If a strain is rescued by a signalling compound, the represented protein can be considered to function upstream of that signalling molecule (figure 4.7). As there are strains that presented a rescued phenotype with both 8-Br-cAMP and FK506 it suggests calcineurin inhibition feeds in above the cAMP signal. Additionally, there were strains that presented a rescued phenotype with FK506 but not 8-Br-cAMP; this suggests that calcineurin inhibition also feeds in downstream of the cAMP signal.

4.4: Conclusions

The addition of potential signalling compounds to the growth media of the non-invasive and poorly-invasive strains identified in chapter 3 has led to a number of suggestions about the signalling events that take place during invasion.

1) cAMP is involved in signalling for invasion.
2) cAMP is also involved in signalling for filament formation and this signal is separable from the signal for invasion.
3) Iron stimulated invasion may intervene after the cAMP signal
4) Calcineurin is a negative regulator of invasion.
5) cAMP signalling may result in calcineurin repression to elicit an invasive response.
6) Calcineurin inhibition may regulate multiple stages of the invasive signalling process.

This method of investigating the role of signalling compounds has provided insights into the regulation of invasion. Therefore, this same method may be equally applicable to the regulation of the tertiary stage of invasion, filament formation. This is will be investigated in chapter 5.

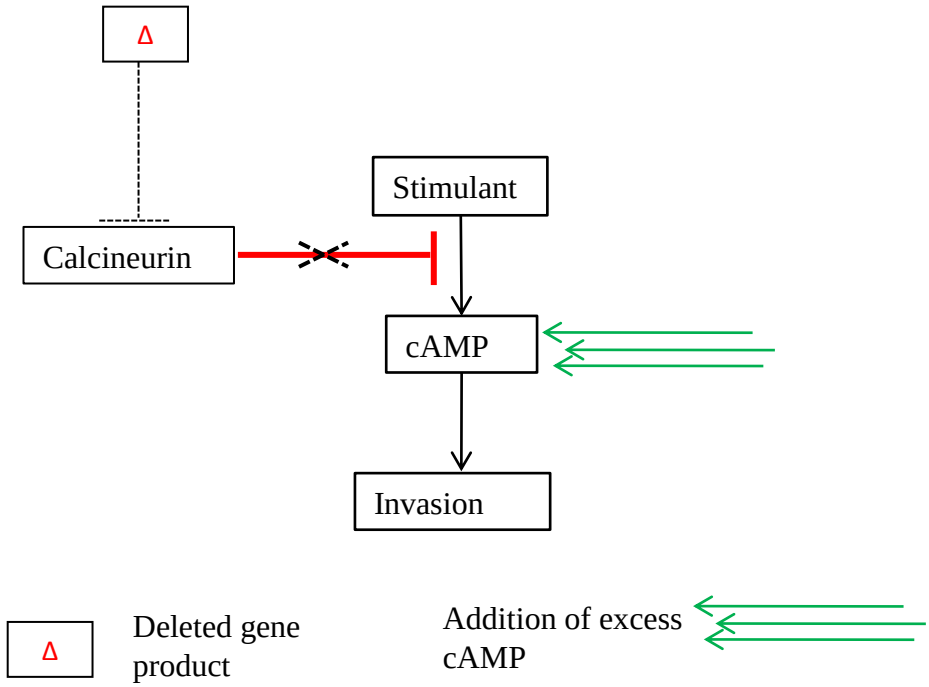


Figure 4.8 The rescue of a strains aberrant invasive phenotype by FK506 and cAMP. The deleted gene product (Δ) normally functions to inhibit calcineurin so it is unable to repress invasion (black dashed line). When the gene is deleted, the calcineurin inhibition no longer takes place which results in repression of invasion (solid red line). Addition of cAMP rescues the aberrant invasive phenotype as calcineurin inhibition may feed in above the cAMP signal.

Chapter 5: Signal transduction during filament formation.

5.1: Signalling for filament formation

The data presented in chapter 3 allowed the description of a tertiary stage of the invasive process. This has been named filament formation and is proposed to be a unique combination of monopolar growth and cellular extension. Filament formation shares similarities with hyphal formation in pathogens such as *C. albicans* where the stage is considered important in virulence (Sudbery et al. 2004) . Therefore, understanding the signalling events during filament formation may contribute to development of antifungal drugs. Additionally, the tertiary stage of the invasive process may involve a transition from bipolar to monopolar growth so study of this stage will be insightful into the regulation of polarised growth. The identification of class Ia strains that cannot form filaments provide an excellent tool for the investigation of signalling events that regulate this process.

5.1.1: Class Ia strains cannot form filaments

Filament formation results from monopolar growth and cellular elongation, therefore the class Ia strains cannot complete one or both of these events. This may result from a defect in the growth machinery or an inability to signal to the machinery. This chapter investigates the signalling processes that take place during filament formation. The data presented in chapter 4 implicates signalling pathways that may be involved in invasion. These may also be involved in filament formation. cAMP is a signalling compound whose production is activated by high glucose and this signalling cascade is required for invasion (Amoah-Buahin et al. 2005). Calcineurin is a protein phosphatase, the deletion of which results in hyper-invasion (chapter 3) and excess iron is an artificial activator of the invasive response (Prevorovsky et al. 2009). Additionally, pyridoxal-5-phosphate has been implicated in filament formation due to the aberrant morphology of *spcc18.10Δ.* Spcc18.10 is a pyridoxal kinase responsible for the production of pyridoxal-5-phosphate.

These four signalling compounds were tested on class Ia strains and *S. pombe* 972 to determine what, if any, role they may have in filament formation.

5.2: Results

Nine strains were designated as class Ia in the LNB screen (chapter 3). These were unable to form filamentous protrusions. Growth on invasion-inducing media for 7 days produces optimal filament morphology for *S. pombe* 972 (Amoah-Buahin et al. 2005),

therefore this is the growth period used in this work. The class Ia phenotype could arise from a delay in filament formation. To investigate this, strains were grown over longer periods of time.

5.2.1: Class Ia phenotype is not a result of slow filament formation

To ensure the class Ia phenotype did not result from a delay in filament formation the class Ia strains were grown for 30 days on invasion-inducing media. This is over four-times the normal growth period. No differences were observed between the filament morphologies after 7 and 30 days (data not shown). Therefore the class Ia aberrations arise from defects in the formation of the filaments and not the time required for filament formation.

5.2.2: Class Ia strains exhibited variable phenotypic severity

The nine strains within this phenotypic group do not all exhibit the phenotype with equal severity (table 3.7, figure 5.1).

The existence of this phenotype implies that a signal is required for the tertiary stage of the invasive process (figure 5.2).

This chapter focuses on what this signal might be. cAMP signalling is required for the secondary stage of the invasive process (Amoah-Buahin et al. 2005), and has been shown to affect the filament morphology (Chapter 4) (Prevorovsky et al. 2009).Therefore, its role during filament formation was investigated via growth of class Ia strains next to a filter disc soaked in 0.1M 8-Br-cAMP, a cAMP analogue.

5.2.3: 8-Br-cAMP stimulates filament formation

Growth next to a filter disc soaked in 0.1M 8-Br-cAMP resulted in an extreme morphological alteration for *alg10*Δ*, spcc18.10*Δ*, fkh2Δ* and *kes1Δ* (figure 5.3, table 5.1) and a partial alteration for *asl1Δ* (figure 5.3 table 5.1). *Spcc18.10*Δ*, fkh2Δ, kes1Δ* and *alg10*Δ were able to form filamentous protrusions that were indistinguishable from *S. pombe* 972 on the same media; these exhibited dramatically elongated filamentous cells (figure 5.3). *Asl1*Δ was capable of forming filaments but the cells within these filaments were not elongated as seen for the afore mentioned strains (figure 5.3). The invasive efficiency of these strains was also higher on 8-Br-cAMP media (figure 5.4). 8-Br-cAMP did not alter the morphology of *tea1*Δ*, tip1*Δ*, tea2*Δ or *mbo1*Δ. Growth with 8-Br-cAMP clearly separates class Ia into two subgroups, those that are affected by 8-Br-cAMP and those that are not. This confirms the conclusion from Chapter 4 that

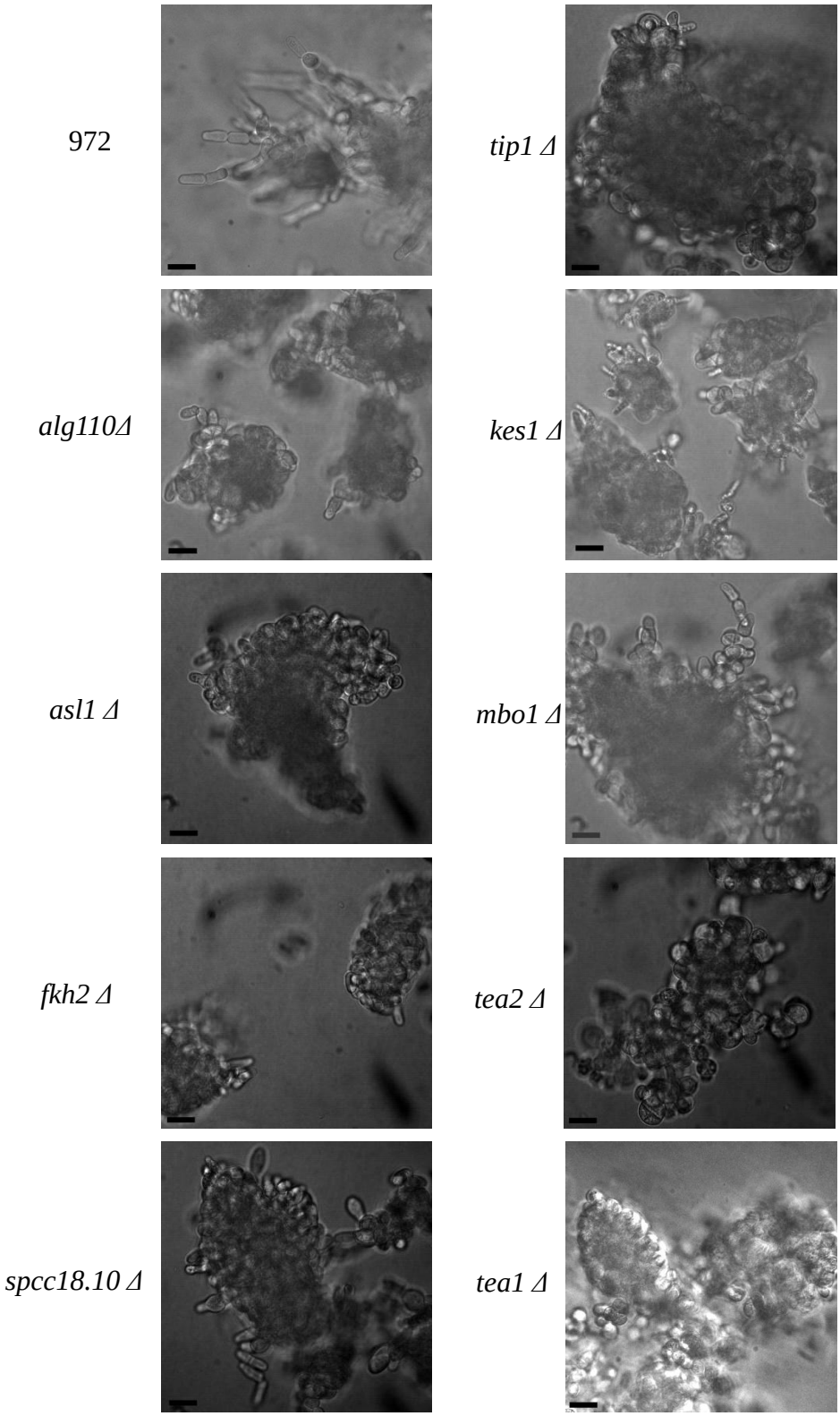


Figure 5.1 Class Ia strains that were unable to form filamentous protrusions. Strains exhibit the morphological defect with varying severity, table 3.7.

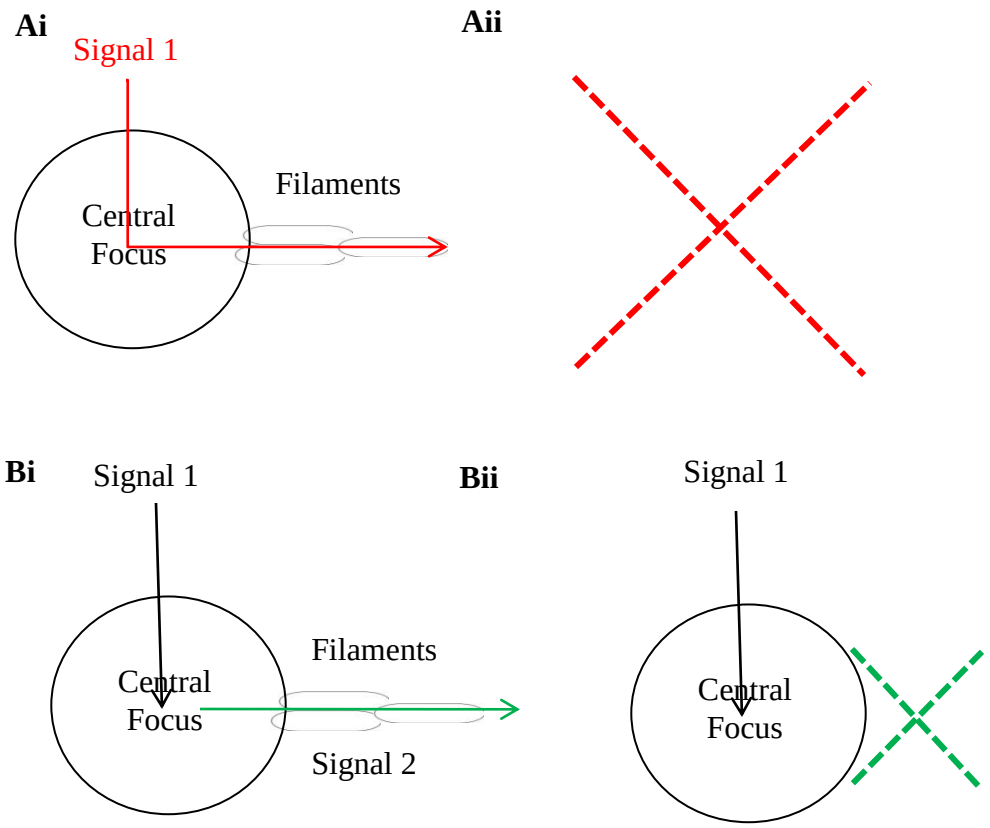


Figure 5.2 The invasive process is regulated by at least two separable signals. If there were one signal that caused invasion and filament formation then one event would not be separable from the other. In Ai, signal 1 causes both invasion and filament formation, therefore when it is removed (Aii) neither can be seen. If invasion and filament formation are caused by separate signals (Bi) then removal of signal 2 (Bii) would still allow invasion, but not filament formation.

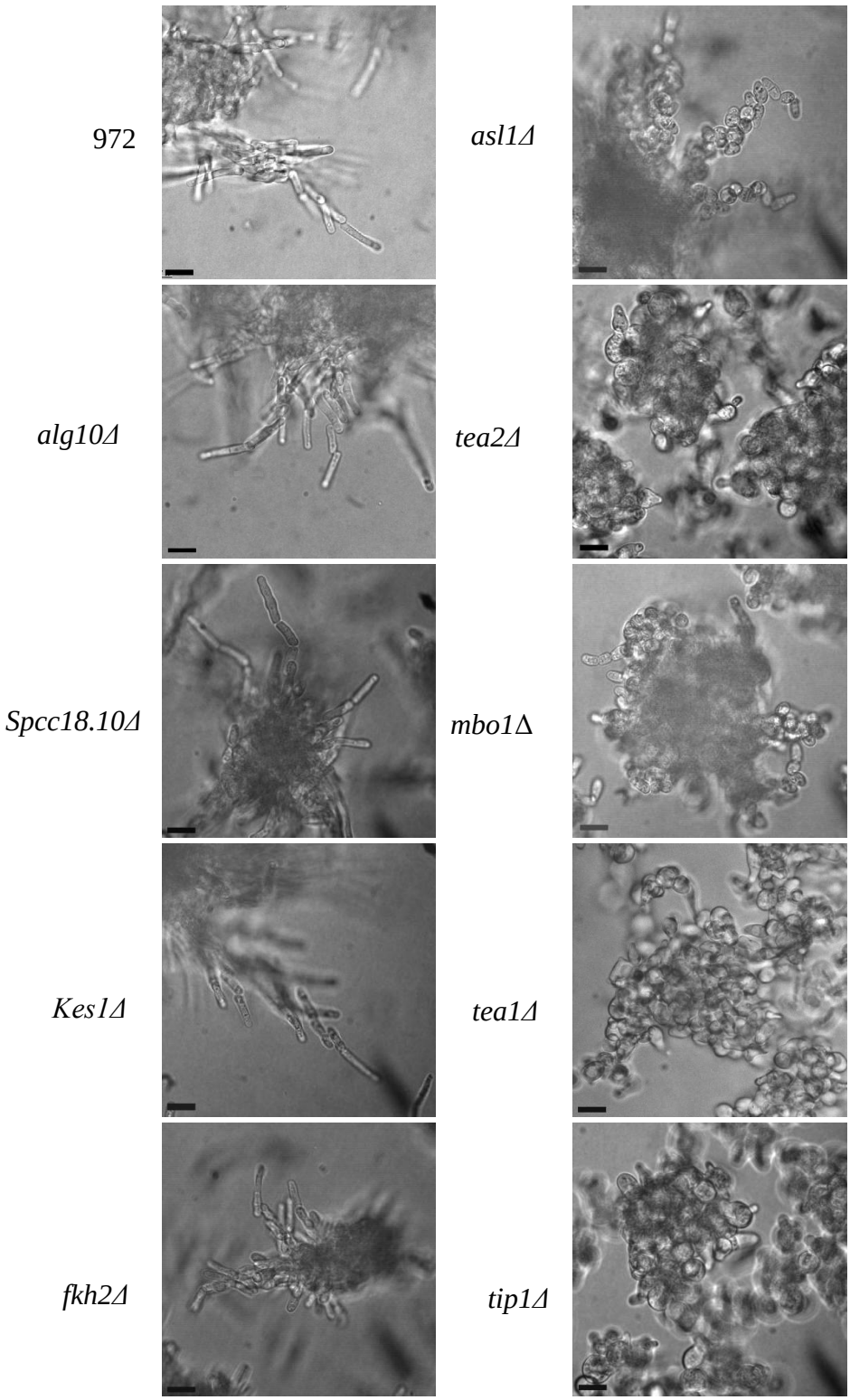


Figure 5.3 The effect of 0.1M 8-Br-cAMP on Class Ia strains. 8-Br-cAMP rescues filament formation of *alg10Δ*, *spcc18.10Δ, kes1Δ* and *fkh2Δ*. It partially alters filament formation in *asl1Δ* but does not alter filament formation in *tea1Δ,tip1Δ*, *tea2Δ* or *mbo1Δ* . Bar10µm.

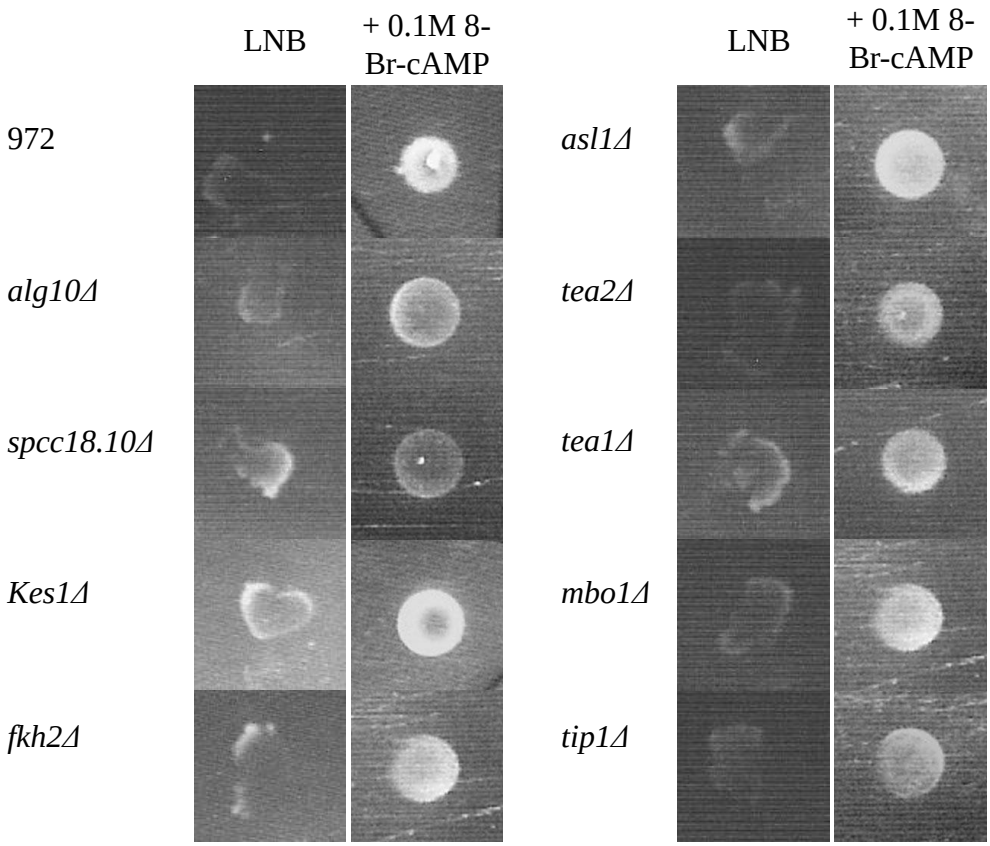


Figure 5.4 The effect of 0.1M 8-Br-cAMP on invasive efficiency of Class Ia strains. All class Ia strains show increased invasion with 8-Br-cAMP.

Gene Name	Gene Description	Growth on 8-br-cAMP	Growth on FK506
Mto1	Involved in microtubule cytoskeleton organization and biogenesis	No morphological rescue	No morphological rescue
SPCC18.10	Pyridoxine-pyridoxal-pyridoxamine kinase	Morphological rescue	Morphological alteration
Tea2	Kinesin-like protein, involved in the localization of polarity factors	No morphological rescue	No morphological rescue
Tea1	Microtubule-associated protein, involved in cell-end anchoring of growth polarity determinants	No morphological rescue	No morphological rescue
Alg10	Involved in N-linked glycosylation	Morphological rescue	Morphological alteration
Tip1	Involved in microtubule stabilisation and targeting to the cell ends	No morphological rescue	No morphological rescue
Asl1	O-glucosyl hydrolase	Partial morphological rescue	Partial morphological alteration
Kes1	Oxysterol binding protein	Morphological rescue	Morphological alteration
Fkh2	Fork head transcription factor	Morphological rescue	Morphological alteration

Table 5.1 The effect of 8-br-cAMP and FK506 on morphology of class Ia strains.

cAMP signalling has an effect specific to filament formation that is separable from its affect on invasion.

The results from addition of 8-Br-cAMP to the growth conditions confirmed that this method is an efficient way of investigating potential signalling compounds. Therefore this approach was used to investigate the role of calcineurin, which may function as a negative regulator of filament formation.

5.2.4: Calcineurin is a negative regulator of filament formation

The strain representing the calcineurin B regulatory subunit (*Spcc830.06Δ*) was classified as hyper-invasive in the LNB screen (chapter 3). Additionally, addition of a calcineurin inhibitor (FK506) to the growth medium has been shown to affect invasive efficiency of non-invasive strains (chapter 4). Therefore, calcineurin may play a negative role in the invasive process. FK506 is a calcineurin inhibitor so addition of FK506 to growth media mimics deletion of calcineurin B.

To investigate the role of calcineurin during filament formation 0.5µg/ml FK506 was added to the growth medium of the class Ia strains. Table 5.1 describes the effect of FK506 on the invasive phenotype of class Ia (figure 5.5). *Tea1*Δ, *tip1*Δ, *mbo1*Δ and *tea2*Δ did not exhibit a morphological alteration when grown on 0.5µg/ml FK506 media (figure 5.5). *Fkh2Δ, kes1Δ, alg10Δ* and *spcc18.10Δ* exhibited a dramatic morphological alteration (figure 5.5). On 0.5µg/ml FK506 LNB media, *fkh2*Δ, *kes1*Δ, *alg10*Δ and *spcc18.10*Δ are capable of forming extended filamentous protrusions that radiate out from the central focus. These filamentous protrusions appear the same as those of wild type *S. pombe* 972 on 0.5µg/ml FK506 media. They exhibit filamentous cells that do not appear to complete cytokinesis (figure 5.5). This lack of complete septation is due to calcineurin's involvement in cytokinesis (Yoshida et al. 1994). *Asl1*Δ exhibited a partially altered morphology on 0.5µg/ml FK506 media. Filaments start to form, but do not extend very far (figure 5.5). The lack of septation is also seen in *asl1Δ*.

0.5µg/ml FK506 LNB media causes hyper-invasive efficiency of the class Ia strains (figure 5.6).

This result suggests that calcineurin is a negative regulator of filament formation. The final signalling compound to be tested for its effect on filament formation due to its effect on invasion is iron.

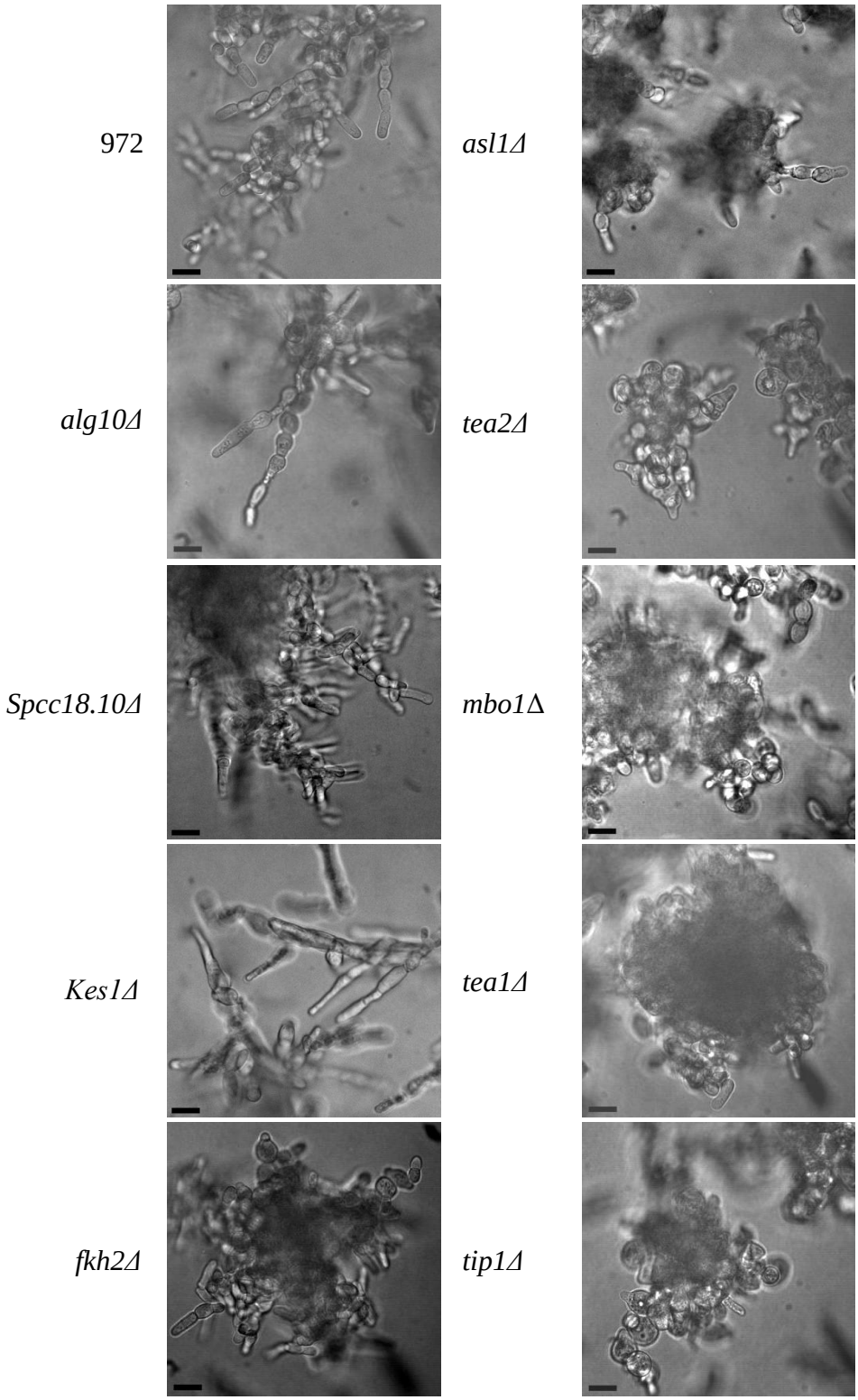


Figure 5.5 The effect of 0.5µg/ml FK506 on filament formation of Class Ia strains. FK506 alters filament formation in *alg10Δ, spcc18.10Δ, kes1Δ* and *fkh2Δ*. It partially alters filament formation in *asl1Δ* but does not alter filament formation in *tea1Δ,tip1Δ, tea2Δ*, or *mbo1Δ*. Bar 10µm

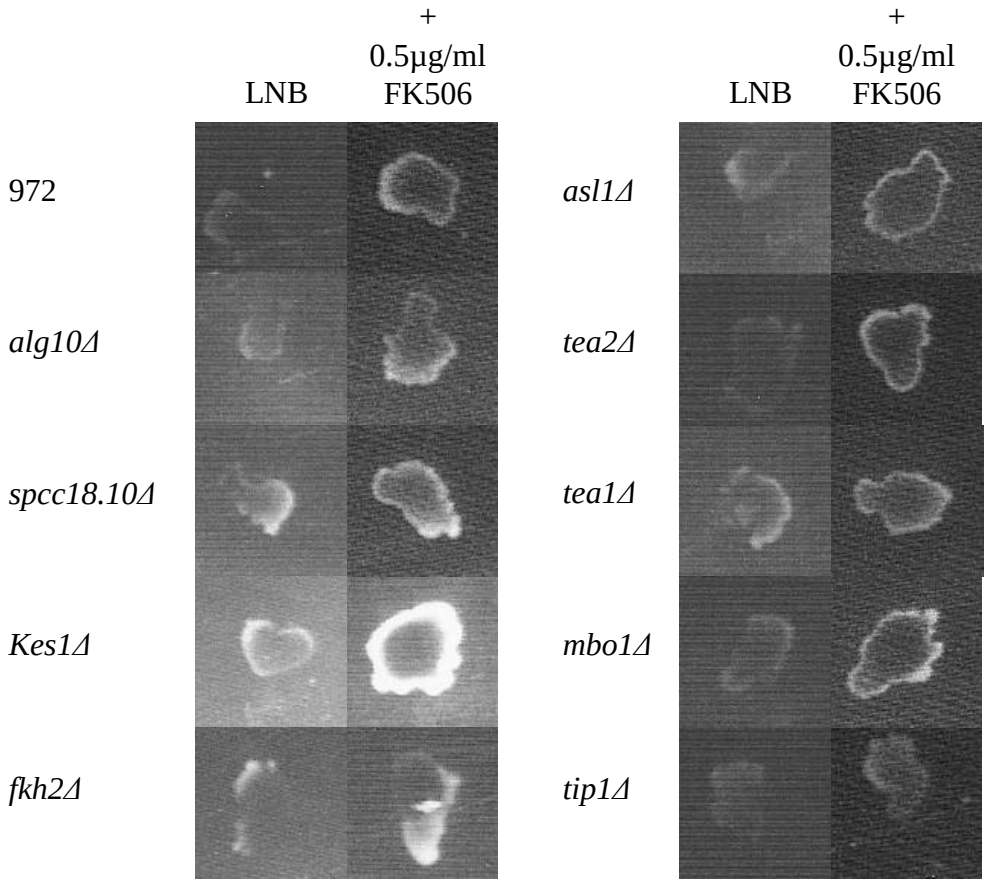


Figure 5.6 The effect of 0.5µg/ml FK506 on invasive efficiency of class Ia strains. FK506 increases invasive efficiency.

5.2.5: 4mM $FeCl_2$ causes an aberrant invasive phenotype

High environmental iron has been implicated as a promoter of the invasive process (Prevorovsky et al. 2009). Additionally Fep1, an iron-sensing transcriptional regulator (Pelletier et al. 2002), has been implicated in invasion due to the poorly invasive phenotype of its representative strain in the LNB screen (chapter 3). The affect of iron on filament formation can be tested by addition of $FeCl_2$ to the growth media of class Ia strains.

The addition of 4mM $FeCl_2$ to the growth media has a dramatic effect on the morphology of all the class Ia strains as well as *S. pombe* 972 (figure 5.7). Many invasive foci are formed: however the strains are unable to form filamentous protrusions. Additionally, the peripheral cells of the foci appear extremely bulbous and dead (figure 5.7).

Despite the morphological effect, all class Ia strains exhibited a hyper-invasive phenotype on 4mM $FeCl_2$ supplemented growth media (figure 5.7).

The previous experiments with $FeCl_2$ and 8-Br-cAMP have shown that addition of signalling compound to growth media is an efficient method for analysing their role during filament formation. The phenotype exhibited by *spcc18.10Δ* suggests pyridoxal-5-phosphate may be a signalling compound during filament formation (chapter 3, section 3.3.4if) so this was further investigated.

5.2.6: Pyridoxal-5-phosphate does not alter the invasive morphology of *spcc18.10Δ*

Spcc18.10 is responsible for production of pyridoxal-5-phosphate (P-5-P) directly from pyridoxal and indirectly from pyridoxine and pyridoxamine (Ike J. Jeon 1995). *Spcc18.10Δ* is a class Ia strain and therefore is implicated in control of filament formation. The morphological defect displayed by *spcc18.10Δ* may result from absence of the protein itself or the product it produces. Therefore, the role of P-5-P in filament formation was investigated by addition of P-5-P to the growth media of *spcc18.10Δ* and *S. pombe* 972.

Three different concentrations (1μM, 20μM and 100 μM) of P-5-P were added to the growth media of *S. pombe* 972 and *spcc18.10Δ*. Neither the invasive efficiency nor filament morphology of *S. pombe* 972 or *spcc18.10Δ* was affected by P-5-P (figure 5.8). This suggests that it may be Spcc18.10 function that is important for filament formation. Spcc18.10 phosphorylates pyridoxal to form pyridoxal-5-phosphate. It also

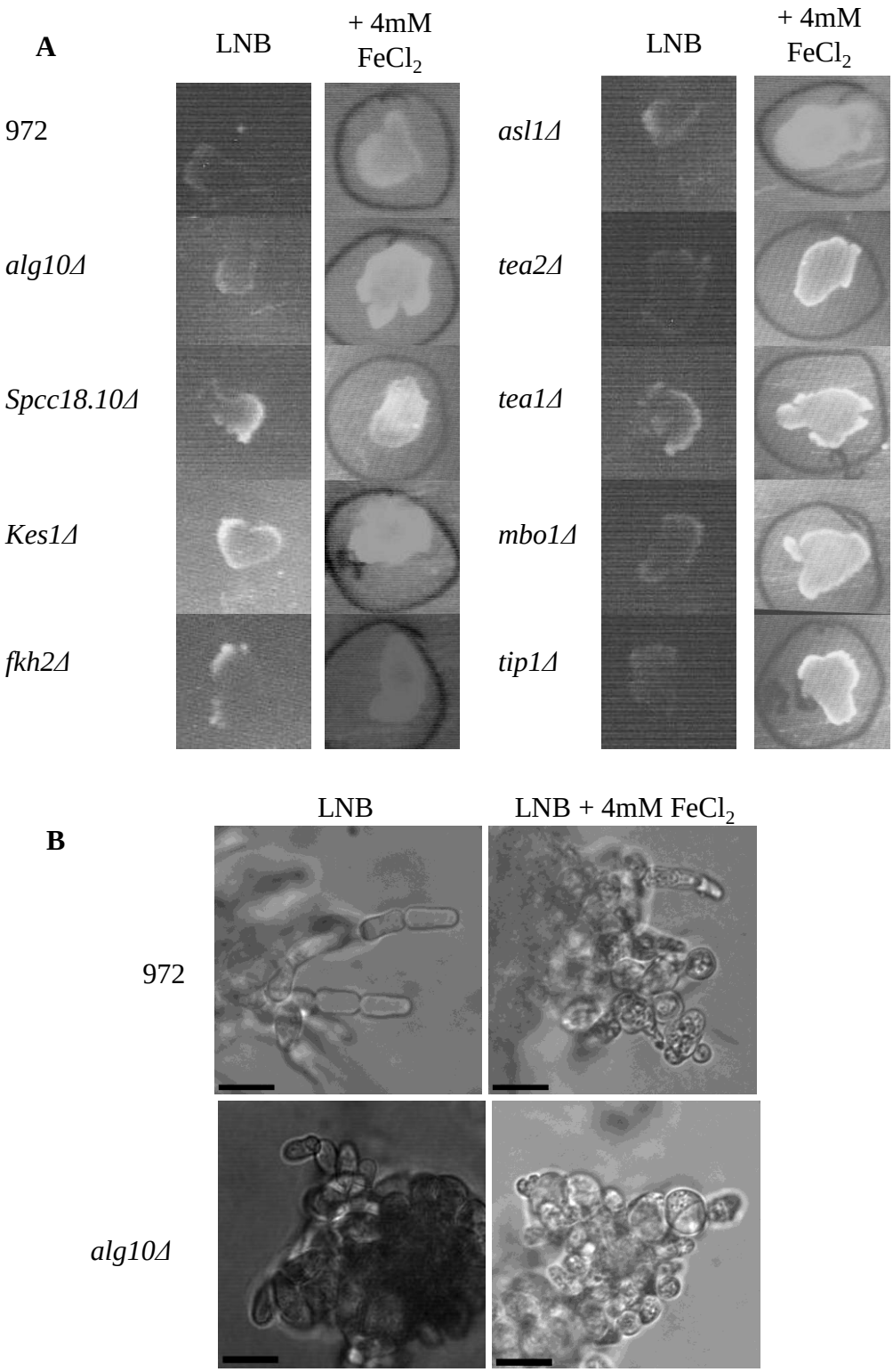


Figure 5.7 The effect of 4mM $FeCl_2$ on A) invasive efficiency and B) filament formation of *S.pombe* 972 and class Ia strains, represented by *alg10 Δ* in B. A) 4mM $FeCl_2$ increases invasive efficiency B) Morphology of both 972 and *alg10 Δ* is grossly aberrant on 4mM $FeCl_2$ containing media.

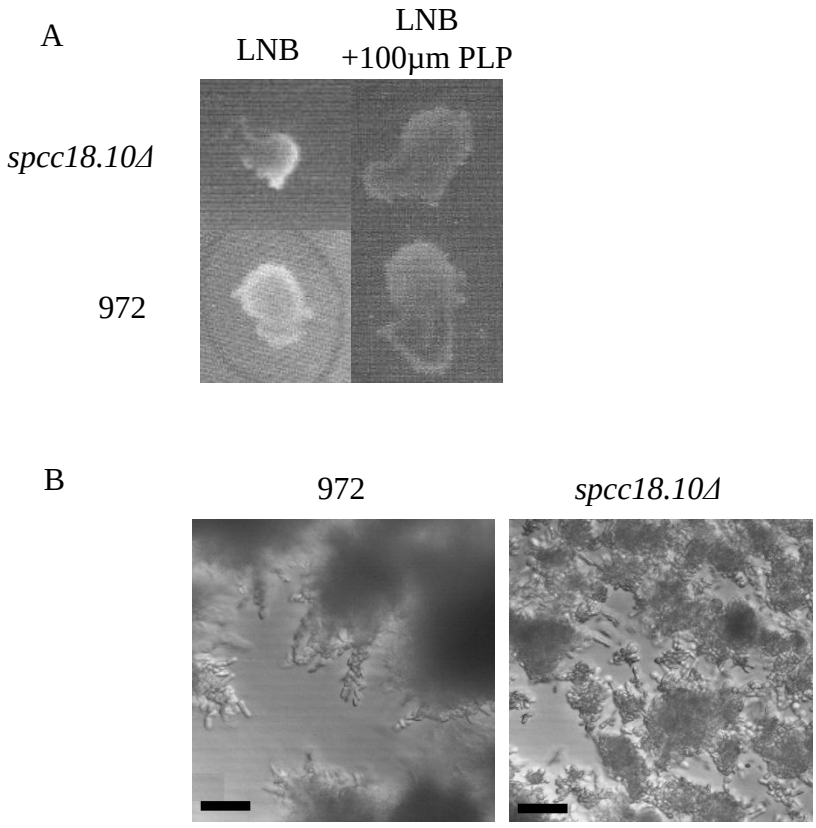


Figure 5.8 The effect of 100µM P-5-P on (A) Invasive efficiency and (B) filament formation of 972 and *spcc18.10Δ*. Bar 50µm.

phosphorylates pyridoxine and pyridoxamine; these products then require further modification to be converted to pyridoxal-5-phosphate. Potentially, these intermediate products, pyridoxine-5-phosphate and pyridoxamine-5-phosphate, could be the compound(/s) involved in filament formation. Alternatively, the phenotype may arise from a build up of Spcc18.10 substrate.

5.2.6i: Pyridoxal does not alter invasive morphology of *spcc18.10Δ*

The absence of Spcc18.10 from a cell will not only result in reduced product, but also increased substrate levels (pyridoxal, pyridoxine and pyridoxamine). The build up of substrate could lead to inhibition of filament formation (as discussed in chapter 3.3.4if). To analyse the effect of substrate on filament formation, the morphology of wild type *S. pombe* 972 was analysed on 100µm pyridoxal containing media. This had no inhibitory effect on filament formation (data not shown).

Addition of exogenous pyridoxal and pyridoxal-5-phosphate did not affect filament morphology of *S. pombe* 972 or *spcc18.10Δ.* This suggests it is Spcc18.10 that functions during filament formation and not its product or substrate. The localisation of proteins can be informative with regards to their function therefore Alg10 and Spcc18.10 were localised during filament formation.

5.2.7: Alg10-GFP and Spcc18.10-GFP localised to the same areas in filaments as they did in single cells

Alg10 and Spcc18.10 may be involved in signalling during filament formation as their aberrant phenotypes can be rescued by addition of exogenous signalling compounds. Localising proteins can aid in identifying their cellular function, therefore the localization of Alg10 and Spcc18.10 was analysed in filaments using GFP tagged proteins. Plasmids encoding the GFP tagged proteins were purchased from Riken (http://dna.brc.riken.jp/en/yoshidayeast_en.html) and expressed as chromosomal integrants (Materials and Methods).

The localization of both Agl10-GFP and Spcc18.10-GFP appear the same in filaments as they do in single cells. Alg10-GFP localised to the cell membrane and endoplasmic reticulum and Spcc18.10 appears to localise to the cytoplasm and the nucleus (figure 5.9).

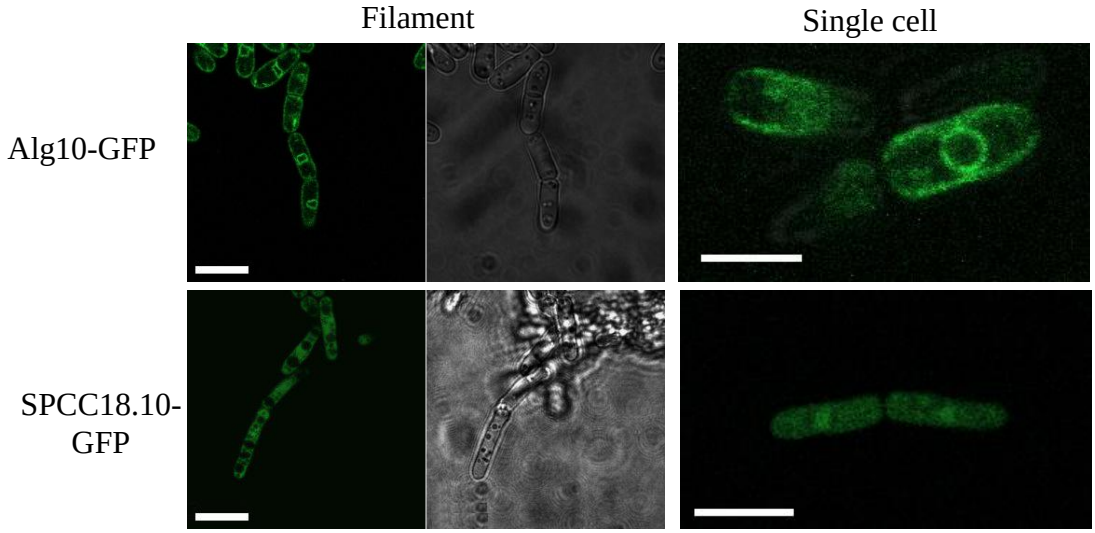


Figure 5.9 Localisation of SPCC18.10-GFP and Alg10-GFP in single cell and filaments. Bar 10µm.

5.3: Discussion

5.3.1: Variation in phenotypic severity of class Ia strains

The nine strains of class Ia which were unable to form filamentous protrusions exhibit the phenotype with differing severities (table 3.6). Filament formation is achieved by consistent monopolar growth and cellular elongation. The most extreme phenotypes were exhibited by *tea1Δ tea2Δ, tip1Δ* and *mbo1Δ*. Tea1, Tea2, Tip1 and Mbo1are proteins that form part of the growth polarity machinery (Browning et al. 2000; Heitz et al. 2001; Busch et al. 2004). Lack of these proteins means the consistent monopolar growth site cannot be established so regardless of any signalling processes, the machinery is not present to produce monopolar growth. In this sense they can be considered effectors of the cAMP signal. The remaining five strains (*kes1Δ, alg10Δ, fkh2Δ, asl1Δ* and *spcc18.10Δ*) of class Ia do not have immediately obvious roles as effectors, so may be involved in signalling during filament formation. They exhibit slightly less severe phenotypes than the effectors, probably because it is easier to by-pass a defect in signalling for a response than it is to bypass a defect in effecting a response.

Class Ia may represent proteins involved in signalling of filament formation. Therefore, various potential signalling compounds were analysed for their effect on the class Ia strains. The first signalling candidate to be discussed is 8-Br-cAMP.

5.3.2: The role of cAMP during filament formation

The role of cAMP signalling during filament formation was investigated via growth of strains with a cAMP analogue, 8-Br-cAMP.

5.3.2i: A cAMP signal is required for the tertiary stage of the invasive response

Growth with 0.1M 8-Br-cAMP rescues the morphological defects displayed by *fkh2Δ, alg10Δ, spcc18.10Δ* and *kes1Δ* and partially rescues the *asl1Δ* defect but does not rescue the defects of *tea2Δ, tea1Δ, tip1Δ* and *mbo1Δ*. This implies a cAMP signal is required to stimulate filament formation. Additionally, all class Ia strains are hyper-invasive upon treatment with 0.1M 8-Br-cAMP. This confirms a cAMP signal also stimulates the secondary stage of invasion, however the cAMP effects on the secondary stage and tertiary stage of invasion are separable (figure 5.2).

5.3.2ii: High levels of cAMP in the leading filament may be achieved by asymmetric membrane inheritance

The phenotype displayed by *kes1Δ, spcc18.10Δ, alg10Δ, fkh2Δ* and *S. pombe* 972 when grown with 0.1M 8-Br-cAMP on LNB is not the same as *S. pombe* 972 invasive growth on LNB. Upon treatment with 0.1M 8-Br-cAMP all cells in the filaments become elongated in comparison to only the end filament cell on LNB. In Chapter 3 (section 3.3.4ib) the leading filament cell was proposed to be the only cell growing in a monopolar, elongated pattern. As all filamentous cells appear to grow this way on 0.1M 8-Br-cAMP media it suggests that during filament formation intracellular cAMP only increases in the leading cell of the filament. As *kes1Δ, spcc18.10Δ, alg10Δ* and *fkh2Δ* are unable to form the leading filament on LNB media but can do so with 0.1M 8-Br-cAMP it suggests the represented proteins may function to increase intracellular cAMP in the leading cell of the filament.

cAMP production is stimulated by high glucose through the Git3 membrane bound receptor (Welton et al. 2000). The interaction of glucose with the extracellular ligand binding domain of Git3 causes indirect activation of intracellular Cyr1 (adenylate cyclase) and cAMP production (Hoffman 2005). Invasion-inducing media has a high glucose content therefore cAMP production will be active during invasion. To achieve a higher cAMP signal in the leading filament the membrane-bound Git3 and Cyr1 may be asymmetrically segregated during the cell cycle to ensure the leading filament inherits more of these proteins which would lead to more cAMP production in the lead filament (figure 5.10). This biased segregation of cAMP production proteins may be coupled with down regulation of their genes in the secondary cells to result in an increased level of cAMP production machinery in the leading cell of the filament compared to the secondary cells of the filament. The asymmetry of these proteins may be a result of specific plasma membrane organisation as well as transcriptional up-regulation in the leading filament tip. How might Kes1, Fkh2, Alg10 and Spcc18.10 function in this proposed mechanism for cAMP increase?

5.3.2iii: Proposed roles of Fkh2, Alg10, Kes1 and Spcc18.10 during filament- associated cAMP signalling

Kes1 is proposed to be an oxysterol binding protein based on its homology to *S. cerevisiae* KES1 (Kozminski et al. 2006). In *S. cerevisiae* oxysterol binding proteins have been shown to contribute to organization of membrane components (Kozminski et

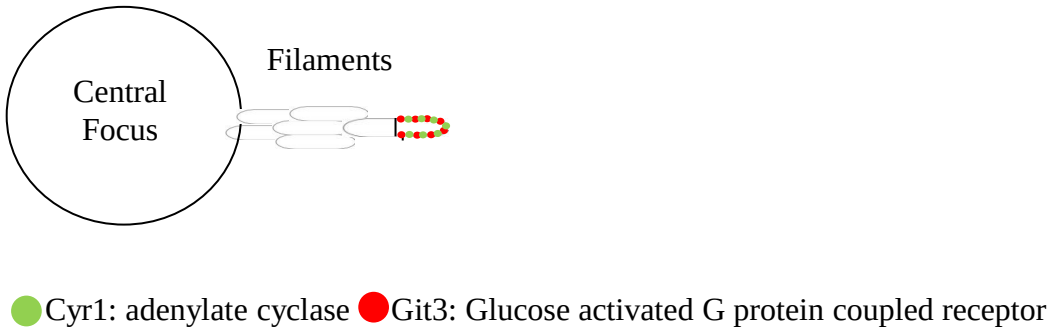


Figure 5.10 During filament formation Git3 and Cyr1 may be asymmetrically localised to the leading filament cell prior to division, to achieve a higher level of cAMP production in that leading filament only.

al. 2006). Maybe Kes1 helps to asymmetrically localise Git3 and Cyr1 to the leading filament (figure 5.10). This could result in a significant increase in cAMP production resulting in the unique growth pattern of the leading filament.

Alternatively, cAMP may rescue the *kes1Δ* aberrant phenotype if Kes1 is involved in cellular elongation during filament formation. *Sc.*Kes1 has been shown to affect Rho1 and Cdc42 signalling (Kozminski et al. 2006). If the same is true in *S. pombe* Kes1 may be involved in cellular elongation by inhibiting Rho1, which is proposed to be a negative regulator of filament formation (chapter 3). In this situation, the requirement for Kes1 to delay mitosis via Rho1 could be over-riden by cAMP which delays mitosis by an alternate route. Kes1 localises to the dividing septum in single cells (http://www.riken.jp/SPD/36/36H03.html). This localisation may change during filament formation allowing Kes1 to organise plasma membrane-bound proteins.

Alg10 was outlined as a potential effector of filament formation in Chapter 3 (section 3.3.4ic). Effectors are responsible for implementing growth and would not be expected to be rescued by the addition of signalling compounds. As the filament formation defect of *alg10*Δ can be rescued by growth with 0.1M 8-Br-cAMP, it seems unlikely it is an effector. Alg10 is proposed to be responsible for the rate limiting step in N-linked glycosylation based on its homology to *S. cerevisiae* ALG10 (Burda et al. 1998). N-linked glycosylation is a post-translational modification of many cell surface proteins (Mora-Montes et al. 2009). Potentially, the modification of cell surface proteins aids with the asymmetric membrane organisation of Git3 and Cyr1 as proposed in figure 5.10. Alternatively, Alg10 may be involved in delaying mitosis. A previous report on mammalian cells linked delay of mitosis with N-linked glycosylation (Larsson 1985). If a similar mechanism is in place during filament formation of *S. pombe* the rescue of the defect by cAMP may be over-riding the normal function of Alg10.

Fkh2 is a transcription factor that inhibits genes responsible for cytokinetic commitment (Bulmer et al. 2004). The failure of *fkh2Δ* to form filaments probably results from filaments going through premature cytokinesis and not being able to elongate (Chapter 3, section 3.3.4ie). Treatment of single cells with a cAMP analogue leads to mitotic delay (Kishimoto et al. 2000), therefore the rescue of *fkh2Δ* phenotype probably arises from over-riding the failure to delay cytokinesis with a delay of mitosis.

It is un-clear how Spcc18.10, a pyridoxal kinase, may be involved in producing an increased cAMP signal in the leading filament.

Analysis of the strains that regained the ability to form filaments with 0.1M 8-Br-cAMP has allowed the proposal of a mechanism for how the signal is generated, but how does this signal result in filament formation?

5.3.2iv: Pka1 mediated mitotic delay may cause cellular elongation during filament formation

Filament formation is achieved by cellular elongation and monopolar growth. This is probably achieved by a combination of signalling pathways. As 8-Br-cAMP rescues the aberrant phenotypes of these strains it suggests the cAMP signal may be involved in both of these events. Cellular elongation of *S.pombe* 972 is also displayed by single cells upon growth with exogenous cAMP and is caused by Pka1 mediated delay on mitosis (Kishimoto et al. 2000). This is probably also the case in filament formation. Monopolar growth is not exhibited by single cell *S. pombe* 972 on 8-Br-cAMP implicating cAMP maintenance of monopolar growth during filament formation is unique. The class Ia strains that did not show regain of filament formation (*mto1Δ, tea1Δ, tea2Δ* and *tip1Δ)* or only show partial regain of filament formation (*asl1Δ)* with 0.1M 8-Br-cAMP may represent proteins involved in monopolar growth.

5.3.2v: Asl1 may remodel the cell wall in response to an increase in intracellular cAMP

*As1l*Δ partially regained the ability to form filaments when grown with 0.1M 8-Br-cAMP. Growth may be monopolar but the site of growth is not as well defined as in *S. pombe* 972 and the other class Ia strains. The cells appear to direct growth to the correct cell end, but the exact position of growth site is not consistent as seen for the other class Ia strain and *S. pombe* 972 with 8-Br-cAMP (figure 5.11). Additionally the filamentous cells are not elongated in *asl1Δ* foci. This suggests that Asl1 functions to effect the cAMP signal resulting in growth site determination and cellular elongation. Asl1 is a cell wall protein that may be involved in re-modelling the cell wall to adapt to changes in the environment (de Groot et al. 2007). This suggests remodelling the cell wall is required for cAMP mediated control of filament formation.

5.3.2vi: Tea2, Tea1, Tip1 and Mbo1 are effectors of filament formation

Tea2Δ, tea1Δ, tip1Δ and *mbo1Δ* did not regain the ability to form filaments when grown with 8-Br-cAMP. Tea1 and Tip1 have been shown to exhibit monopolar localisation at

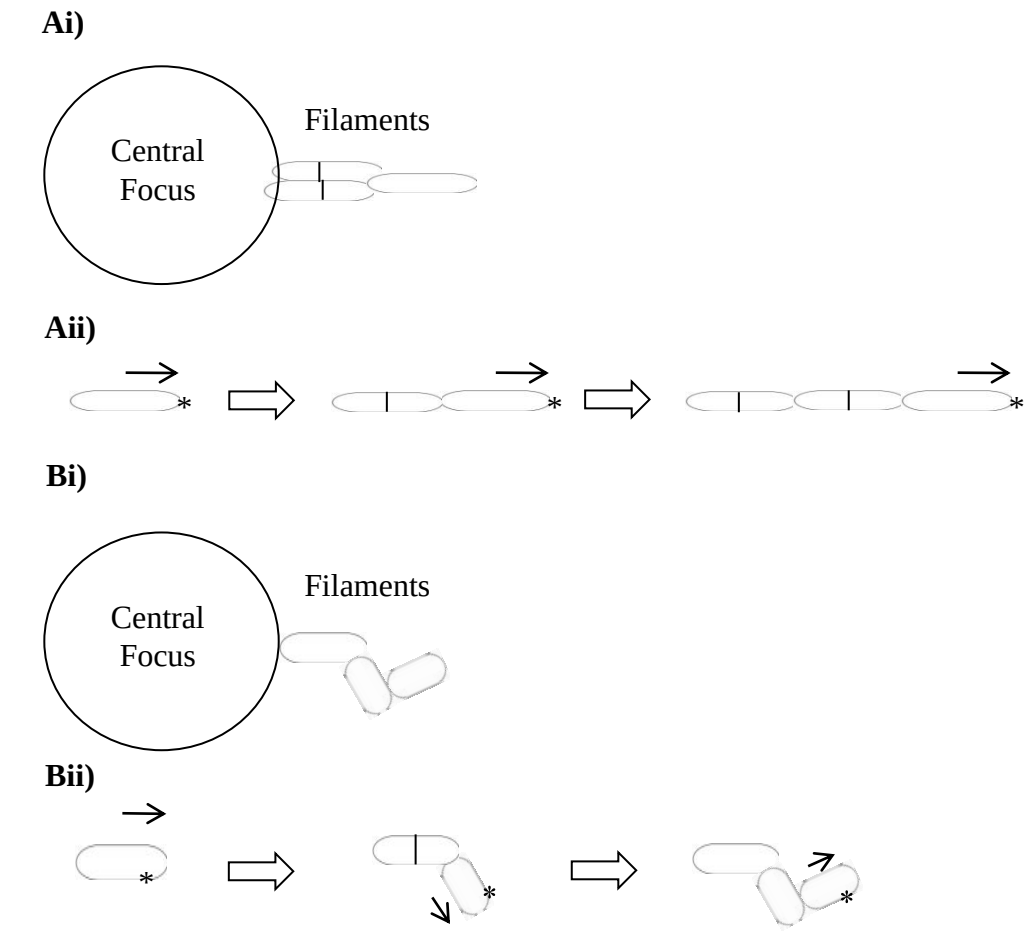


Figure 5.11 Mis-positioning of *asl1Δ* growth site. Ai) Schematic of *S. pombe* 972 invasive focus. Aii) During filament formation, monopolar growth is restricted to the growing filament half and the specific growth site is restricted to the tip of the filament. Bi) Schematic of *asl1Δ* invasive focus. Bii) During filament formation monopolar growth is still restricted to the growing filament half, however the specific growth site is no-longer restricted to the cell tip and is mis-positioned resulting in filaments that grow in odd directions.

the non-growing filament end during filament formation (Dodgson et al. 2010). However, Tea1 was shown to be transported to the growing end but it does not remain there (Dodgson et al. 2010). This implies the re-arrangement of localisation of these proteins is crucial for filament formation. The cAMP signal may be partially responsible for this re-arrangement to enable filament formation. Lack of these proteins means the consistent monopolar growth site cannot be established so regardless of any signalling processes, the machinery is not present to produce monopolar growth. In this sense they can be considered effectors of the cAMP signal.

5.3.2vii: Conclusions from experiments with 8-Br-cAMP

The analysis of the 8-Br-cAMP effect on the morphologies of class Ia strains has lead to a number of propositions about filament formation.

1) A cAMP signal is required for filament formation and this signal is separable from the signal required for invasion.

2) cAMP levels may be higher in the leading filament cell, which could be achieved by asymmetric inheritance of Git3 and Cyr1.

3) Kes1 and Alg10 may be involved in the regulation of asymmetric Git3 and Cyr1 inheritance.

4) Mitotic delay probably causes cAMP mediated cellular elongation during filament formation.

5) Microtubule associated transport proteins and Asl1 are effectors of the cAMP filament formation signal.

5.3.3: Calcineurin signalling during filament formation

To investigate the role of calcineurin in filament formation, class Ia strains were grown on 0.5μg/ml FK506 media. This resulted in an alteration of filament morphology for *kes1Δ, alg10Δ , fkh2Δ, spcc18.10Δ* and *asl1Δ* but not *tea1Δ, tea2Δ, tip1Δ mbo1Δ*. The filaments of the affected strains, as well as that of *S. pombe* 972 were multi-septate and did not appear to complete cytokinesis. This effect of FK506 has been described in single cells and is due to calcineurin-mediated regulation of cytokinesis (Yoshida et al. 1994) implying this same regulation takes place during filament formation. Alongside this previously described morphological defect that arises from calcineurin inhibition, the class Ia aberrant morphology is rescued and strains are capable of consistent monopolar growth resulting in filament formation. The rescue of this defect by growth

on 0.5µg/ml FK506 media suggests calcineurin is a negative regulator of filament formation. The inhibitory effect of calcineurin on filament formation suggests it may be a target for regulating the tertiary stage of the invasive response.

5.3.3i: Kes1, Alg10, Fkh2 and Spcc18.10 may repress calcineurin to allow filament formation

Calcineurin may be active in the cell to repress filament formation until the cell is specifically stimulated to form filaments. When this signal is received the cell may repress calcineurin activity to allow filament formation. Kes1, Alg10, Fkh2 and Spcc18.10 may be responsible for the inhibition of calcineurin to allow filament formation. As such, in *kes1*Δ, *alg10*Δ, *fkh2*Δ and *spcc18.10*Δ cells no such inhibition is taking place and so calcineurin continues to repress filament formation. Growth on FK506 media represses calcineurin therefore it no longer represses filament formation in *kes1*Δ, *alg10*Δ, *fkh2*Δ and *spcc18.10*Δ. Calcineurin is a Ca^{2+} dependent phosphatase (Yoshida et al. 1994) so perhaps these proteins (Kes1, Alg10, Fkh2 and Spcc18.10) influence intracellular Ca^{2+} to inhibit calcineurin function.

Alternatively calcineurin-mediated inhibition of filament formation could function antagonistically to a pathway involving Kes1, Alg10, Fkh2 and Spcc18.10.

The phenotypes of *kes1*Δ, *alg10*Δ, *fkh2*Δ and *spcc18.10*Δ on 0.5µg/ml FK506 media suggest they function upstream or antagonistically to calcineurin. Conversely, *asl1Δ* displays a phenotype that suggests it may function downstream of calcineurin.

5.3.3ii: Asl1 may function as an effector of cellular elongation

Growth of *asl1Δ* on 0.5µg/ml FK506 media results in a subtle alteration of filament morphology. Filaments start to form but do not protrude far away from the central focus. In chapter 3 *asl1Δ* morphology was attributed to defects in monopolar growth site determination. As *asl1Δ* filaments begin to protrude away from the central focus on 0.5µg/ml FK506 media it suggests the monopolar growth defect has been rescued. However, the strain cannot elongate, suggesting the cellular elongation defect of *asl1Δ* has not been rescued. Failure to rescue the cellular elongation defect suggests Asl1 may be an effector of this event in filament formation. This correlates with the *asl1Δ* phenotype displayed on 0.1M 8-Br-cAMP media.

The phenotypes displayed by *tea2Δ, tea1Δ, tip1Δ* and *mbo1Δ* on FK506 media also correlate with the phenotype displayed on 0.1M 8-Br-cAMP media.

5.3.3iii: Tea2, Tea1, Tip1 and Mbo1 are effectors of filament formation

Tea2Δ, tea1Δ, tip1Δ and *mbo1Δ* did not regain the ability to form filaments on FK506 media. The phenotype displayed by these strains correlates with the suggested role of the represented proteins as effectors of filament formation (see section 5.3.2vi). Growth of class Ia strains on FK506 media induces a hyper-invasive phenotype of all strains. This correlates with the data of Chapter 4 and suggests calcineurin functions at the secondary stage of the invasive process as well as the third.

5.3.3iv: Conclusions from experiments with FK506

The rescue of class Ia morphological defect by growth on 0.5µg/ml FK506 media has led to a number of propositions:

1) Calcineurin is an inhibitor of filament formation.

2) Fkh2, Kes1, Alg10 and Spcc18.10 may function as upstream inhibitors of calcineurin.

3) Asl1 may function as an effector of cellular elongation during filament formation.

5.3.4: Growth on 4mM $FeCl_2$ causes aberrant invasion

Growth of all class Ia strains and *S. pombe* 972 on 4mM $FeCl_2$ media, which confers high extracellular iron, results in grossly aberrant foci. This defect may be attributed to a toxic effect of iron. The morphology displayed by class Ia strains and 972 on 4mM $FeCl_2$ media does not correlate with the data presented by Prevorovsky *et al.*(2009), who initially described the induction of invasion by high environmental iron.

5.3.4i: Discrepancies between morphological result may be due to variation between strains

Prevorovsky *et al*. describe the morphology of *S. pombe* grown on 4mM $FeCl_2$ as elongated (Prevorovsky et al. 2009). This does not correlate with the aberrant invasion exhibited here. There are a number of variations between the two bodies of work that could lead to this phenotypic difference. Prevorovsky *et al.* used liquid dots of cell suspensions for growth on 2% agar YES medium for 19 days. This work utilised large clumps of cells for growth on 1% agar LNB medium for 7 days. Finally the *S. pombe* strain used in the studies differed. Out of all these factors, the one most likely to cause the morphological difference noted is the strain used, as it has been documented that different *S. pombe* strains have different invasive potential (Dodgson et al. 2010).

The aim of growing class Ia aberrant strains on 4mM $FeCl_2$ supplemented media was to discern whether or not iron was involved in regulation of filament formation, however the results have been inconclusive.

5.3.4ii: Iron may be involved in regulation of filament formation

The aberrant invasive phenotype displayed by class Ia strains and *S. pombe* 972 on 4mM $FeCl_2$ could theoretically imply that iron is an inhibitor of filament formation. However, the morphology displayed by these strains suggests it is a toxic effect rather than an inhibitory effect.

Growth of class Ia strains on 4mM $FeCl_2$ media has not confirmed or refuted iron as a regulator of filament formation.

5.3.5: The role of pyridoxal-5-phosphate during filament formation

Spcc18.10 is responsible for the production of pyridoxal-5-phosphate (P-5-P), which is a coenzymes in many reactions (Toney 2005). The aim of this experiment was to identify which of these two cellular constituents is required in filament formation. Invasive growth on varying concentrations of P-5-P did not alter filament morphology of *spcc18.10Δ* or *S. pombe* 972. The lack of effect on filament morphology suggests it is Spcc18.10 that is required for filament formation. However, exogenously added P-5-P may not be taken up by the cells, so although these results suggest it is Spcc18.10 that has the functional role, P-5-P cannot be dismissed completely as a factor in filament formation.

5.3.5i: P-5-P may be a regulator of filament formation

S. pombe actively excretes P-5-P from the cell alongside a phosphatase (Chumnantana et al. 2001). This phosphatase removes a phosphate from P-5-P which facilitates cellular uptake. Once taken into the cell it is then re-phosphorylated forming the active P-5-P (Morita et al. 2004). Therefore, addition of P-5-P to the growth media does not necessarily result in higher cellular levels of P-5-P. This suggests the invasive growth results on P-5-P media does not rule out P-5-P as a regulator of filament formation. Alternatively, the morphological defect displayed by *spcc18.10Δ* could result from a build-up of substrate (Chapter 3, section 3.3.4if).

5.3.5ii: Pyridoxal does not inhibit filament formation

Spcc18.10 is a kinase responsible for phosphorylation of pyridoxal, pyridoxine or pyridoxamine (Ike J. Jeon 1995). Potentially it could be a build up of these substrates that repress filament formation. This repression could form part of a negative feedback control mechanism. Invasive growth on pyridoxal containing media did not cause *S. pombe* 972 to exhibit an aberrant phenotype. The lack of effect on *S. pombe* 972 suggests no such inhibition takes place. However, the preferential route for P-5-P production is proposed to be phosphorylation of pyridoxine to pyridoxine-5-phosphate which is then converted to pyridoxal-5-phosphate (Guirard et al. 1988). Pyridoxal and pyridoxamine are converted to pyridoxine for phosphorylation by Spcc18.10. Therefore, the main substrate that would build up in the cell upon deletion of Spcc18.10 would be pyridoxine, which was not tested here.

Invasive growth on P-5-P media is inconclusive with regards to whether the product (P-5-P) or protein (Spcc18.10) is involved in regulation of filament morphology.

5.3.6: The function of Alg10 and Spcc18.10 in filament formation is not obvious from their localisation

Alg10-GFP and Spcc18.10-GFP display the same localisation pattern in filaments as they do in single cells. The cytoplasm of Spcc18.10-GFP cells appears to have some large vacuoles. Large vacuoles have been implicated in filamentous growth of *S. japonicus* (Sipiczki et al. 1998). The large vacuoles of *S.japonicus* are thought to aggregate at the non-growing end, forcing all the growth components to the growing end of the cell. This does not appear to be taking place in *S. pombe* filaments as the large vacuoles are distributed throughout the cell.

The localization of Alg10-GFP and Spcc18.10-GFP has not provided any insight into their role during filament formation.

5.4: Conclusions

Growth of class Ia strains and *S. pombe* 972 on media supplemented with a variety of potential signalling compounds has led to a number of conclusions about the regulation of filament formation.

1) A cAMP signal is responsible for the tertiary stage of the invasive process, but this is separable from the cAMP signal required for invasion.

2) Calcineurin is a negative regulator of filament formation.

3) Tea1, Tea2, Tip1 and Mbo1 are effectors of the cAMP mediated signal.

4) Asl1 may be an effector of cellular elongation.

Chapter 6: Investigation of spindle pole bodies during filamentous growth

6.1: Asymmetry in filaments

Filament formation involves asymmetric growth and effectors of this growth phase exhibit asymmetric localisations (Dodgson et al. 2010). Cellular asymmetry drives differentiation in higher eukaryotes (Bertrand et al. 2009). Filamentous growth may be a unique growth phase (chapter 5), therefore transition between yeast phase and filament formation can be considered as a form of cellular differentiation. Cellular differentiation has been widely studied in higher eukaryotes such as *C. elegans* and *D. melongaster* (Betschinger et al. 2004). These studies suggest differentiation is highly dependent on cellular asymmetry. One such asymmetry involves inheritance of the centrosome, the equivalent to yeast spindle pole bodies (SPB). As centrosome asymmetry is associated with cellular differentiation in higher eukaryotes and asymmetries occur during filament formation, potentially SPB asymmetry may also be involved in filament formation. Additionally, in *S. cerevisiae* the SPB segregation pattern is asymmetrical; the old SPB continually migrates to the daughter bud (Pereira et al. 2001). This localization is dependent on interactions of the microtubules with the bud cell cortex. Delivery of growth components along the microtubules has been shown to be important during filamentous growth, suggesting a similar mechanism may be in place in *S. pombe*.
An initial observation suggested that SPB segregation was biased in filaments and the new SPB consistently migrated to the growing filament end (J. Dodgson, personal communications). This data required extensive affirmation and this is the topic of investigation here. To understand the potential involvement of SPB asymmetry in filament formation the processes associated with the SPB segregation must first be understood.

6.1.1: Spindle pole bodies (SPBs)

Spindle pole bodies (SPBs) are the yeast equivalent to mammalian centrosomes and serve a variety of functions (Pereira et al. 2001). They are the microtubule organising centres for the mitotic spindle and also the site of the septation initiation network (SIN) control and activation (Magidson et al. 2006). Fission yeast have one spindle pole body that duplicates at the G1/S boundary, the SPBs then separate and migrate to opposite ends of the cell to ensure the inheritance of a single SPB by each daughter cell following cytokinesis (Uzawa et al. 2004). The SIN must only be activated from one of these two SPBs to ensure only one division takes place. SIN is activated in late

anaphase and is dependent on a number of proteins (Magidson et al. 2006). The main master regulator of SIN is Spg1 (Krapp et al. 2008).

6.1.2: Spg1 mediated SIN activation

Spg1, a Ras family GTPase, is an SPB associated protein and is the main protein responsible for SIN activation (Schmidt et al. 1997). Spg1 associates to both the old and new SPBs but is only activated on the new SPB (Sohrmann et al. 1998). The activation of SIN is dependent on the guanine nucleotide bound state of Spg1 (Schmidt et al. 1997). If Spg1 is GDP-bound then it is inactive and does not trigger SIN; if it is GTP-bound it is active and triggers SIN. The state of Spg1 is defined by association of GTPase activating and de-activating factors to the SPBs. Cdc16/Byr4 is a GTPase activating complex (GAP) for Spg1. This complex binds to Spg1 in interphase and keeps it in an inactive GDP bound form (Furge et al. 1998). During mitosis Byr4-Cdc16 leaves the SPBs, allowing partial activation of Spg1 (Sohrmann et al. 1998). Full activation is not achieved until Cdk1 activity is lost at anaphase (Guertin et al. 2000). Byr4-Cdc16 then rebinds to the old SPB which recruits Fin1, thus returning it to its inactive state (Grallert et al. 2004).

6.1.3: Fin1 function during SIN

Fin1 is a NIMA kinase that functions downstream of the Cdk1/Cyclin B mitosis promoting factor (MPF) (Grallert et al. 2004). During SIN, Fin1 is recruited to one or both of the SPBs via an age-dependent process. The mechanism of this is not clearly understood but is thought to involve a phosphorylatory event (Grallert et al. 2004). Fin1 is recruited to all old SPBs. It is also recruited to the new SPB in a single cell if the duplicating SPB was itself an old SPB during the previous cell cycle (Grallert et al. 2004). Fin1 associates with the old SPB via Byr4, but it is unclear how it associates with the new SPB. Byr4 is not present at the new SPB as Byr4 functions to inhibit SIN and SIN is activated from the new SPB. Fin1functions to inhibit SIN at the old SPB. In *fin1Δ* cells Cdc7 is recruited to the new SPB as well as the old SPB in 44% of the cells (Grallert et al. 2004).

Fin1 is involved in SIN inhibition, conversely Etd1 is associated with SIN activation and SIN signalling (Garcia-Cortes et al. 2009).

6.1.4: SIN signalling

Spg1 is kept in an active GTP-bound state by Etd1. Etd1 is a proposed Spg1 activating protein, though it is not thought to be a guanine nucleotide exchange factor (GEF) which are normally responsible for activation of GTPases such as Spg1 (Garcia-Cortes et al. 2009). Following Spg1 activation, Cdc7 is recruited to the SPB via Spg1 which subsequently recruits and activates Sid2 facilitating its translocation to the site of septation initiation (Sohrmann et al. 1998; Guertin et al. 2000). Cdc7 is only recruited to GTP-bound Spg1 and is therefore a marker for active Spg1 (Sohrmann et al. 1998). Cdc7.GFP localisation in single cells has shown that initially after SPB duplication Cdc7 associates with both old and new SPB. As mitosis continues, it only localises to one SPB (Sohrmann et al. 1998). Co-localisation experiments with Pcp1.RFP, a marker for the old SPB (Grallert et al. 2004), have shown that Cdc7.GFP consistently localises to the new SPB in single cells. During mitosis Cdc7.GFP (a marker for the SIN activating, new SPB) has been shown to migrate in a non-biased fashion to either the new or old end of the cell (as defined by the previous cell division and birth scar) (Sohrmann et al. 1998), though the evidence for this appears to be only a brief comment, with no quantification of Cdc7.GFP migration.

6.1.5: Aim of chapter 6

To investigate the potential role of SPB asymmetric segregation during filament formation Cdc7.GFP was localised in actively growing filaments. Prior to this, the segregation pattern of SPB in single cells was confirmed.

6.2: Results

All localisation data presented here was carried out in an A1153 strain background. This strain is hyper-filamentous and facilitates live cell imaging of actively growing filaments (Dodgson et al. 2010). All strains were created by mating into the A1153 genetic background and selecting for appropriate progeny (Materials and Methods). These hybrid strains were grown in glass bottom petri dishes which allows microscopy of actively growing filaments.

6.2.1: Segregation of Cdc7.GFP to the old and new end is unbiased in single cell

Cdc7.GFP is a marker for the new SPB which activates SIN (Sohrmann et al. 1998). Previously Cdc7.GFP was stated to segregate in an unbiased fashion to either the old or

new cell end. This previous description (Sohrmann et al. 1998) did not quantify the result, therefore for a comparison of segregation of Cdc7.GFP during filamentous growth, single cell segregation patterns must also be quantified. Additionally the strain used for analysis of the Cdc7.GFP segregation pattern in filaments is a derivative of A1153 strain which was not the strain used by Sohrmann *et al.* (1998).

Cdc7.GFP cells were grown to exponential phase prior to Calcofluor treatment to mark the new and old end of the cell. Calcofluor staining and light transmission methods were used for the differentiation between old and new cell end.

15 cells exhibited visible fluorescent Cdc7.GFP signals, nine of these were noted as migrating to the new end and six to the old end (figure 6.1). The Cdc7.GFP foci were designated as migrating to the old or new end based on which end they were closer to. This data confirm the result published by Sohrman *et al*. (1998), stating that Cdc7.GFP segregation shows no strong bias in single cells, but is this also true in filaments?

6.2.2: Cdc7.GFP localisation in filaments

Single cell growth is bipolar and cells exhibit symmetrical localisation of polarity determining growth factors. In contrast filamentous growth appears to be mono-polar and polarity determining proteins localise asymmetrically. To investigate whether this change in polarisation is mimicked by a change in new SPB segregation, the Cdc7.GFP segregation pattern was analysed during filamentous growth.

6.2.2i: Cdc7.GFP segregation during filamentous growth is biased

22 Cdc7.GFP foci were noted in filamentous cells. 19 of these were seen migrating to the growing filament end (figure 6.2) and three were seen migrating to the non-growing end. This strongly implies there is a bias in Cdc7.GFP migration pattern during filamentous growth of *S. pombe.*

6.2.2ii: Cdc7.GFP migrated to the growing filament end through two consecutive cell divisions

To investigate the possibility that Cdc7.GFP consistently associates with the SPB that migrates towards the growing filament end, time lapse microscopy was utilised with an aim to capture multiple sequential migrations of Cdc7.GFP.

This time lapse captured five filaments going through two cell divisions with the Cdc7.GFP associated SPB migrating to the growing filament end in both anaphase stages for four of them (figure 6.3). In one of the filaments the Cdc7.GFP foci was seen

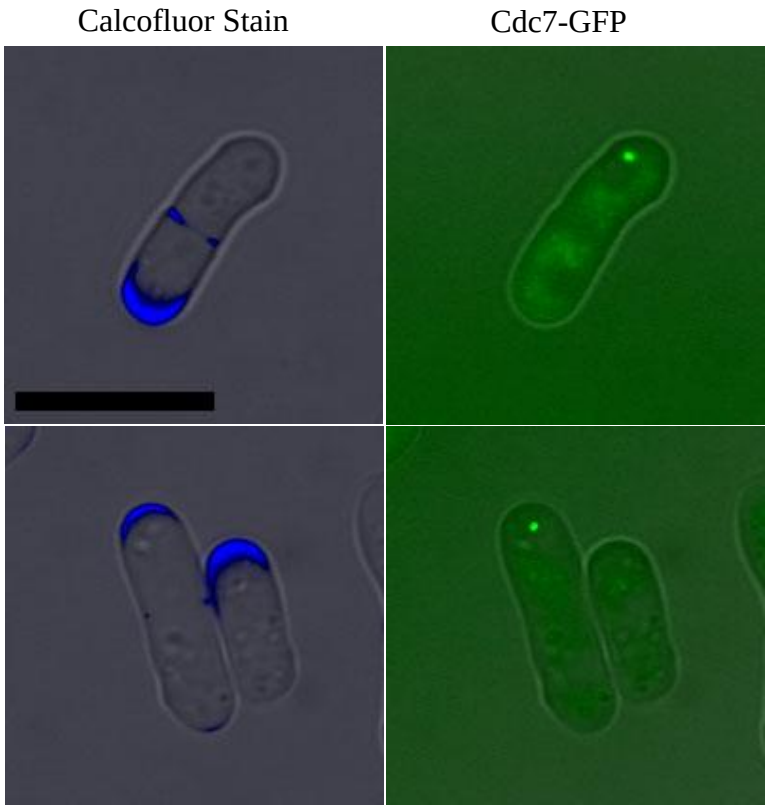


Figure 6.1 Cdc7.GFP bound spindle pole bodies migrate to the old (stained blue in Calcofluor panel) and new end of the single cell in an unbiased fashion. Bar 10μm.

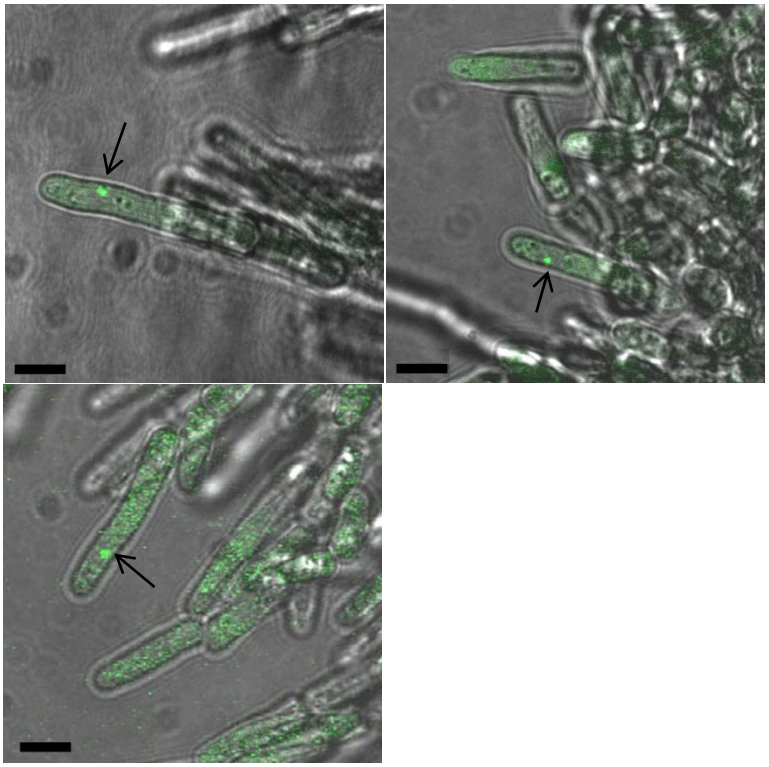


Figure 6.2 Cdc7.GFP migrates to the growing end in filaments. The arrows are pointing at Cdc7.GFP foci. Bar 5μm.

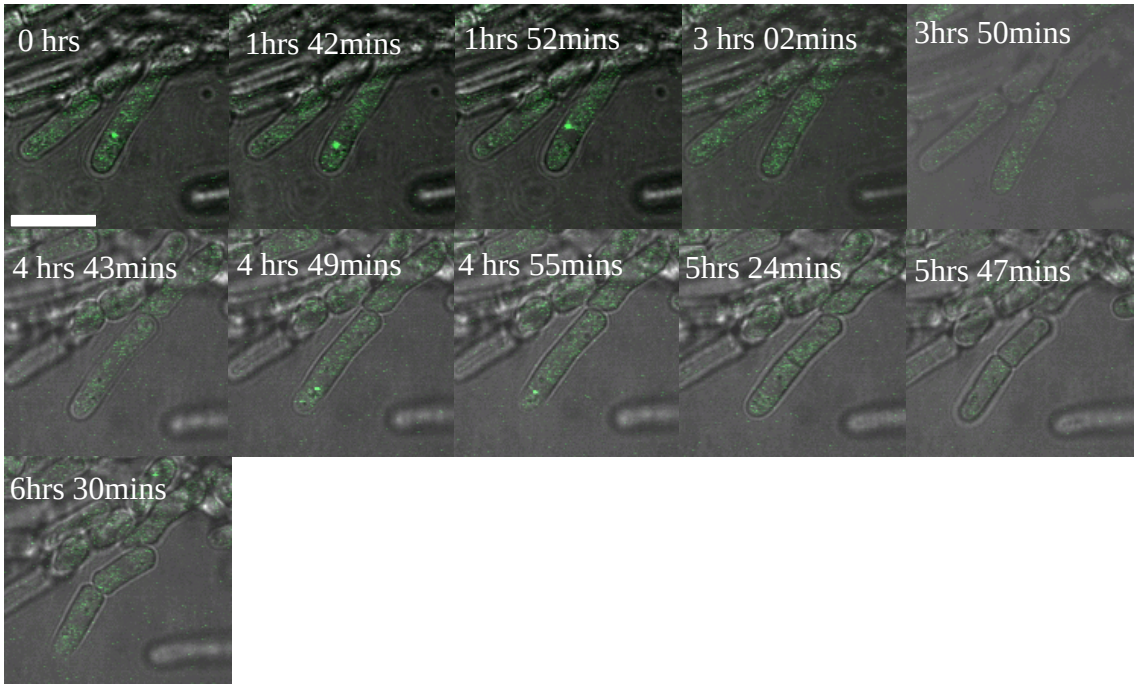


Figure 6.3 Time course images of Cdc7.GFP migrating to growing filament end through two consecutive rounds of cell separation. Time points are estimates. Bar in 0hrs is 10µM.

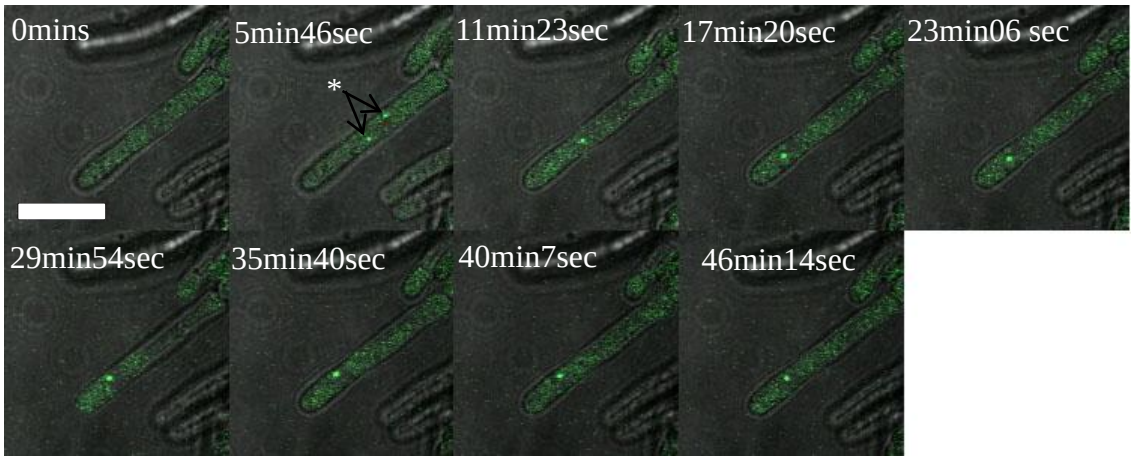


Figure 6.4 Cdc7.GFP is initially present at both SPBs (* arrows in 5min46sec) during early anaphase. As mitosis progresses it is only visible at the SPB migrating towards the growing filament end. Bar 10µM.

migrating to the growing end for the first cell division but then to the non-growing end in the second cell division.

Time lapse microscopy confirms a change in Cdc7.GFP segregation pattern from single cell to filamentous growth. It can also be used to compare the localisation pattern of Cdc7.GFP in single cell and filamentous growth.

6.2.2iii: Cdc7.GFP initially localised to both SPBs during filamentous growth

Time lapse microscopy confirmed the localisation pattern of Cdc7.GFP in filaments mimics that in single cells. Initially Cdc7.GFP localises to both SPBs (* arrows in figure 6.4) but as the SPBs migrate towards their respective pole, Cdc7.GFP signal becomes stronger on only one of the SPBs. In filaments the SPB migrating towards the growing filament end is the SPB which retains the strengthened signal (figure 6.4). These data have outlined the segregation pattern and localisation in the leading filament cell. It can also be used to determine the Cdc7.GFP segregation pattern in the secondary cells.

6.2.2iv: Cdc7.GFP segregation reverted to an unbiased pattern in secondary cells

Secondary cells are directly or indirectly behind the leading filament. These cells appear to revert back to bi-polar growth, as seen in single cells (Dodgson et al. 2010). The migration of Cdc7.GFP was analysed in these cells to see whether it also reverts to the single cell phenotype of unbiased Cdc7.GFP segregation. In the leading filament Cdc7.GFP migrates towards the growing cell end. This end is the old end of the cell. Cdc7.GFP is not seen migrating to the non-growing end of the cell. The non-growing end is the new cell end.

Seven Cdc7.GFP foci were visualised; five of these were seen migrating to the new cell end and two were seen migrating to the old cell end. Figure 6.5 shows migration of the Cdc7.GFP to the old and new ends of secondary cells. This data suggests that the migration of Cdc7.GFP in secondary cells reverts to the unbiased pattern seen in single cells.

The exhibition of a biased Cdc7.GFP segregation pattern during filamentous growth raised the question; what controls this segregation? This was investigated with deletion strains of polarity determining proteins.

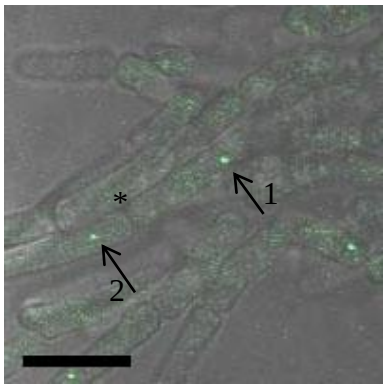


Figure 6.5 Segregation bias of Cdc7.GFP is lost in secondary filamentous cells. Arrows point to Cdc7.GFP foci migrating towards the old and new ends of secondary cells.* marks a septum. This division septum has one SPB moving away from it (arrow 1) and one SPB moving towards it (arrow 2). Irrelevantly of whether * marks a newly formed cell end or is the an old cell end, one SPB is moving away from it and another towards it. Therefore the bias of Cdc7.GFP for the old cell end has been lost in secondary cells. Bar 10µM.

6.2.3: Deletion of polarity components affected Cdc7.GFP segregation during filamentous growth

S.cerevisiae exhibits a biased SPB segregation pattern; the old SPB always migrates to the daughter bud (Pereira et al. 2001). This bias is dependent on microtubule interactions with the bud cell cortex. This interaction is not seen in *S. pombe* during single cell growth. A selection of *S. pombe* cell polarity deletion strains (*tea1Δ, tip1Δ* and *tea2Δ*) which represent proteins associated with the interphase microtubules, are not able to form filamentous protrusions (chapter 3). If a similar mechanism occurs during *S. pombe* filamentous growth as in *S.cerevisiae* single cell SPB inheritance then perhaps the aberrant phenotype displayed by *tea1Δ, tip1Δ, tea2Δ* may be, in part, a result of a failure to direct SPB asymmetry. As Cdc7.GFP is a marker for the new SPB its migration pattern may be affected in the filaments of these deletion strains.
The Cdc7.GFP localisation experiments were performed in a A1153 strain background as this strain is hyper-filamentous (Materials and Methods). As A1153 is hyper-filamentous *tea1Δ, tip1Δ* and *tea2Δ* which were not able to form filaments in a *S. pombe* 972 background (chapter 3) were able to form filaments in the A1153 background but these are morphologically aberrant with many T structures and abnormal branching (figure 6.6a). The hybrid strains were then analysed by confocal microscopy to determine the Cdc7.GFP segregation pattern.

6.2.3i: Deletion of *Tea1* affected Cdc7.GFP segregation pattern

Five Cdc7.GFP foci were visualised in the *tea1Δ*, Cdc7.GFP filaments. Three of these migrated towards the non growing end of the leading filament (figure 6.6a).

6.2.3ii: Deletion of *Tip1* affected Cdc7.GFP segregation pattern

Nine Cdc7.GFP foci were visualised in the *tip1Δ*, Cdc7.GFP filaments. Five of these migrated towards the non growing end of the leading filament (figure 6.6a).

6.2.3iii: Deletion of *Tea2* affected Cdc7.GFP segregation pattern

Seven Cdc7.GFP foci were visualised in the *tea2Δ*, Cdc7.GFP filaments. Two of these migrated towards the non growing end of the leading filament (figure 6.6a).

Pom1Δ, tea4Δ and *fin1Δ* were also analysed for their effect on Cdc7.GFP segregation. Pom1 is a protein kinase that is bi-polarly localised in single cells but mono-polarly localised in filaments (Dodgson et al. 2010). Pom1 affects localisation of proteins at

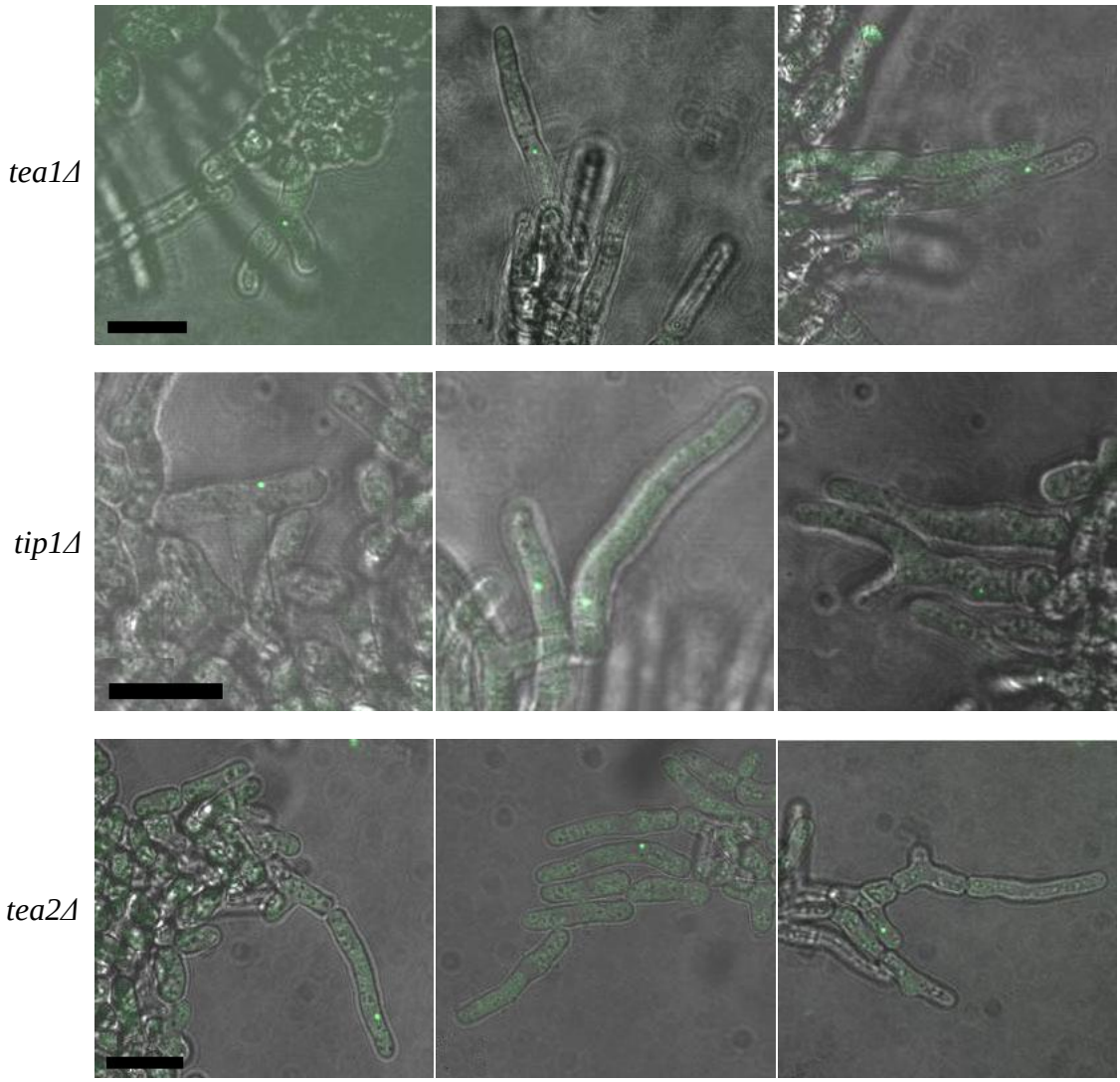


Figure 6.6a Deletion of *tea1, tea2* and *tip1* alters Cdc7.GFP segregation pattern during filamentous growth. Cdc7.GFP foci can be seen migrating to the non-growing end of the cell in *tea1Δ, tea2Δ* and *tip1Δ* strains. Bar 10μm.

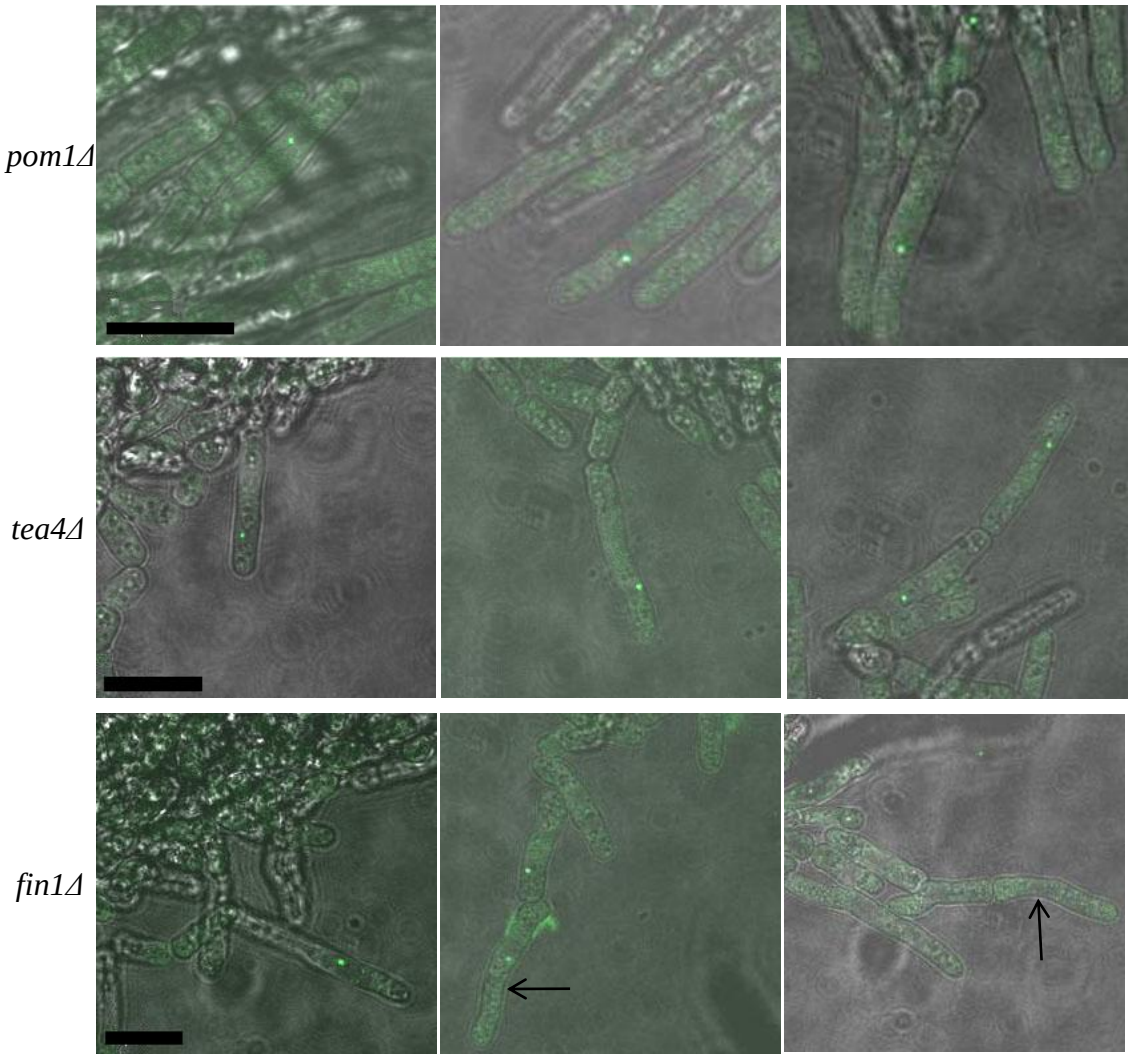


Figure 6.6b Deletion of *tea4*, *pom1* and *fin1* did not alter Cdc7.GFP segregation pattern. Cdc7.GFP can be seen migrating to the growing filament end. Arrows in *fin1Δ* point to where filaments appear to bend, indicating potential problems with growth polarity. Bar 10μm.

cortical nodes that are responsible for the onset of mitosis (Moseley et al. 2009). Wee1 localises to these cortical nodes as well as the SPB (Moseley et al. 2009). If Pom1 affects localisation of factors that associate to the SPB potentially it could affect Cdc7 recruitment to the SPB. Tea4 is a polarity factor required for the localisation of other cell polarity components at the cell tip (Tatebe et al. 2005). If polarity determinants are required for Cdc7.GFP segregation then Tea4 may potentially be involved. Fin1 associates with the old and new SPB and is involved in de-activation of Spg1 (Grallert et al. 2004). As Cdc7 is only recruited to active Spg1, *fin1Δ* may affect the Cdc7.GFP migration pattern. In single cells, *fin1Δ* deletion results in increased Cdc7.GFP association to both SPBs (Grallert et al. 2004). If Cdc7.GFP migration to the filament end is pivotal during filamentous growth then a mutant phenotype may be observed. *Pom1Δ, tea4Δ* and *fin1Δ* were mated to Cdc7.GFP strain and the appropriate progeny were selected. The hybrid strains were then analysed by confocal microscopy to determine the Cdc7.GFP segregation pattern.

6.2.3iv: Deletion of *pom1, fin1* and *tea4* did not affect Cdc7.GFP segregation pattern

Three Cdc7.GFP foci were visualised in the *pom1Δ*, Cdc7.GFP filaments and in the *tea4Δ*, Cdc7.GFP filaments. For each of these strains all three foci migrated towards the growing end (figure 6.6b). Three Cdc7.GFP foci were visualised in the *fin1Δ*, Cdc7.GFP filaments. Two were migrating to the growing end of the filament and one was seen migrating to the non-growing end. Additionally the directionality of growth in these filaments appears slightly aberrant (arrows in figure 6.6b). This suggests Fin1 may be involved in polarity determination during filamentous growth.

There were significantly fewer visible Cdc7.GFP foci in the *tea1Δ, tea2Δ* and *tip1Δ* filaments than visualised in the parental Cdc7.GFP strain. Cdc7.GFP is only visible when associated with Spg1 in the GTP bound state on the SPB. This lack of signal could reflect the importance of these factors in the recruitment of Cdc7.GFP to Spg1. Cdc7.GFP is a marker for the SIN activating SPB (Sohrmann et al. 1998). Spg1 and Fin1 activate and inhibit SIN respectively (Schmidt et al. 1997; Grallert et al. 2004), therefore their localisation may prove insightful to the role of Cdc7.GFP segregation in filamentous growth.

6.2.4: Spg1 and Fin1 localised to SPBs during filamentous growth

The SPB that activates SIN consistently segregates to the growing end of the filament, suggesting a link between filament formation and the SIN. To investigate the role of SIN during filament formation, Spg1, the protein required for SIN activation (Schmidt et al. 1997) and Fin1, a protein involved in SIN inhibition (Grallert et al. 2004), were both localised in filaments. In single cells Spg1 localises to both SPBs after SPB duplication (Garcia-Cortes et al. 2009) whereas Fin1 can associate with one or both SPBs (Grallert et al. 2004).

Spg1.GFP and Fin1.4GFP were crossed into an A1153 strain background (Material and Methods) and actively growing filaments were then used for microscopic analysis of Spg1.GFP and Fin1.4GFP localisation.

Spg1.GFP is visible moving to the growing end of the filament as well as the non-growing rear of a leading filament (figure 6.7a,b,c). Fin1.4GFP gives a very faint signal but can be seen in the front section of the growing filament (figure 6.8 a, b) as well as the rear section of the growing filament (figure 6.8b). One Fin1.4GFP focus cannot be classified as migrating to either end of the filament (figure 6.8c).

6.3: Discussion

Asymmetry of protein localisation may contribute to the regulation of filamentous growth. Therefore the possibility that the SPB segregation was asymmetric was also investigated. Cdc7.GFP was used as a marker for the new SPB and its localisation was tracked in a selection of deletion strains as well as wild type *S. pombe*.

6.3.1: Cdc7.GFP segregation in filaments

6.3.1i: Cdc7.GFP activity in filaments was the same as in single cells

In single cells Cdc7.GFP localises to both SPBs at the beginning of anaphase; as anaphase progresses it is seen at a higher intensity at just one SPB (Sohrmann et al. 1998). This same pattern is seen in filaments (figure 6.4). This suggests the function of Cdc7 is the same in filaments as it is in single cells where Cdc7.GFP binds to the SPB via Spg1-GTP and promotes the septation initiation network (SIN).

6.3.1ii: Cdc7.GFP segregation in filaments was biased

To investigate the possibility that filamentous growth may involve biased Cdc7 segregation Cdc7.GFP localisation was studied. 22 Cdc7.GFP foci were localised in

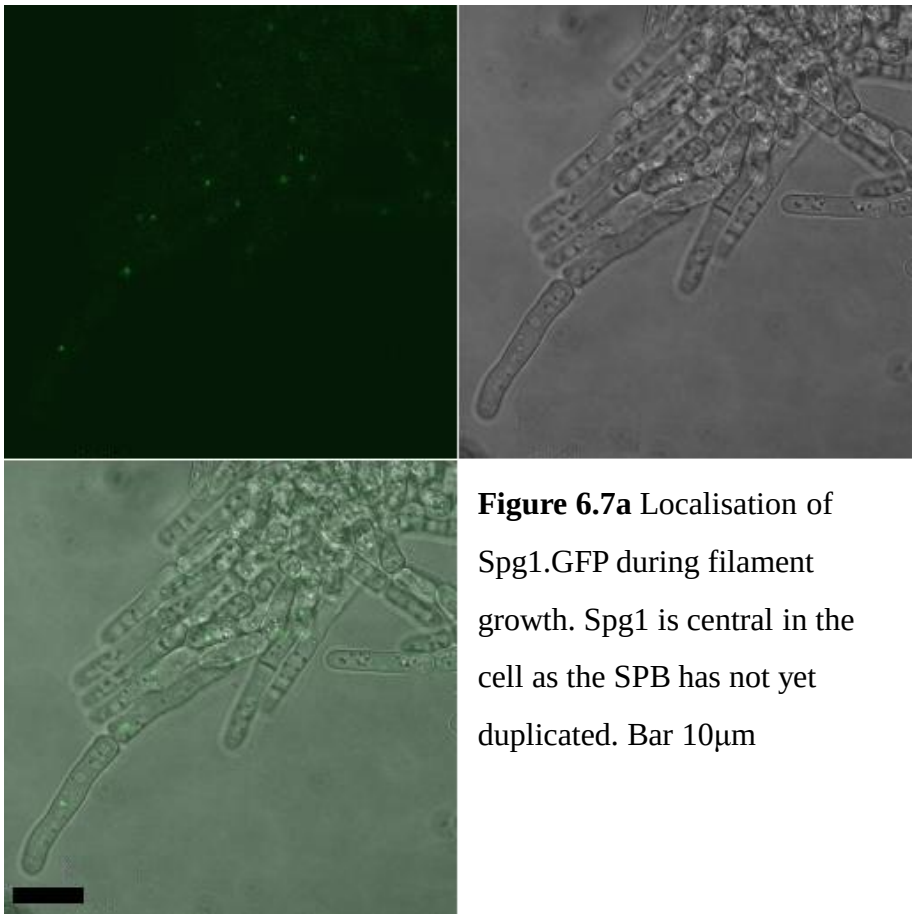


Figure 6.7a Localisation of Spg1.GFP during filament growth. Spg1 is central in the cell as the SPB has not yet duplicated. Bar 10µm

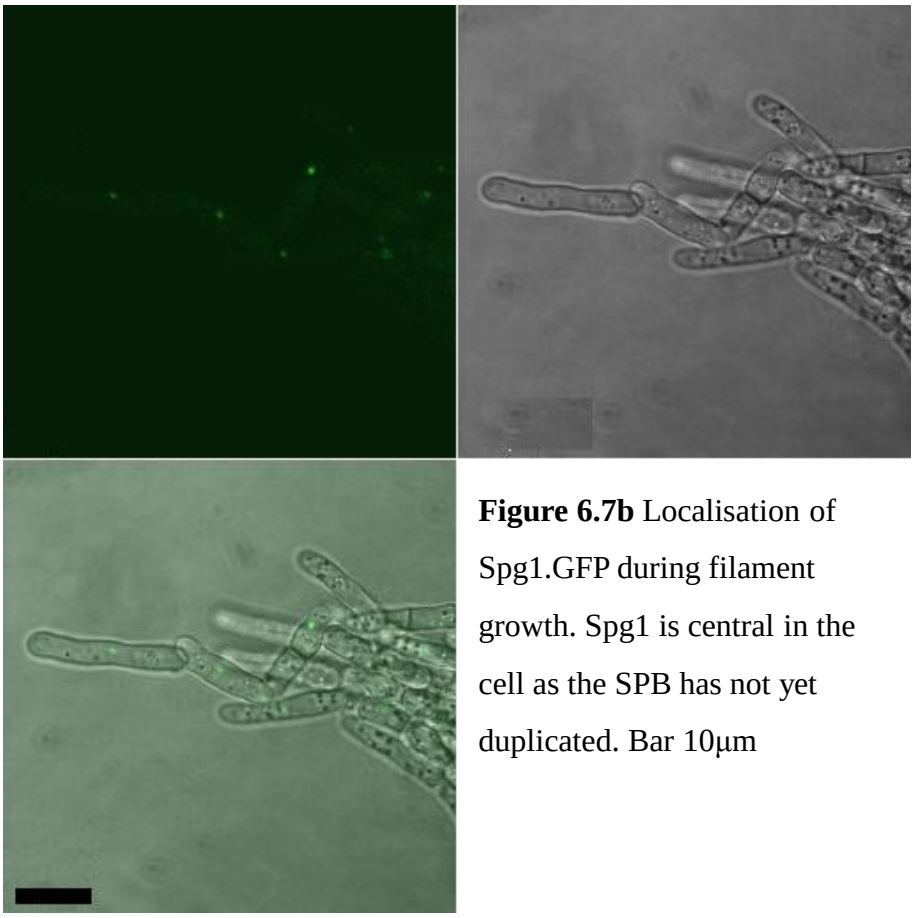


Figure 6.7b Localisation of Spg1.GFP during filament growth. Spg1 is central in the cell as the SPB has not yet duplicated. Bar 10µm

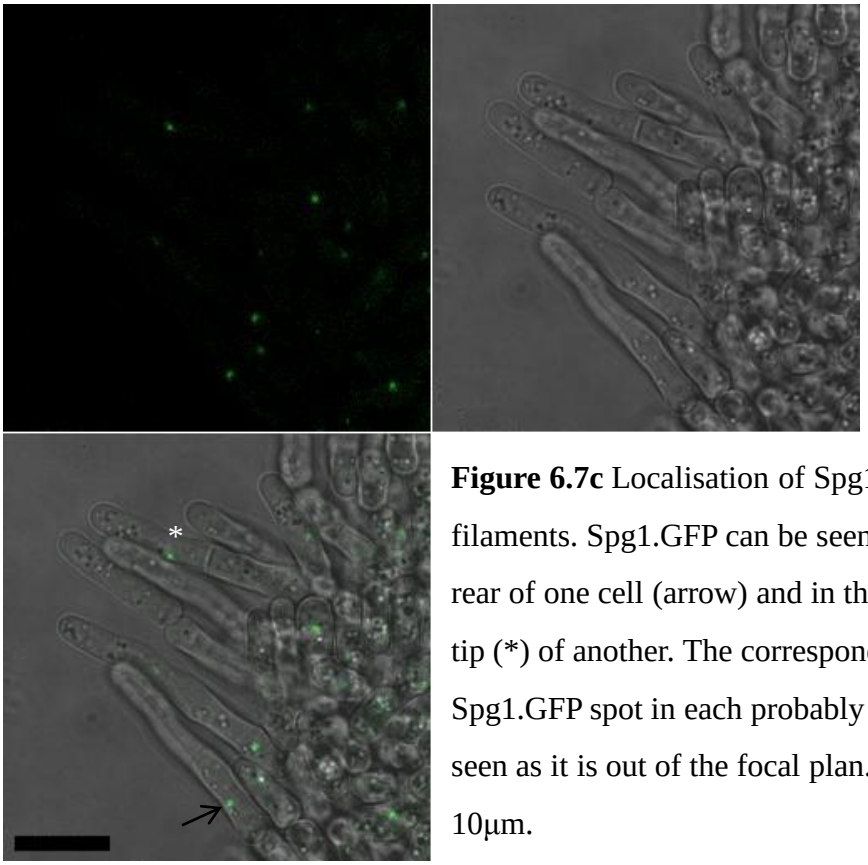


Figure 6.7c Localisation of Spg1.GFP in filaments. Spg1.GFP can be seen in the rear of one cell (arrow) and in the leading tip (*) of another. The corresponding Spg1.GFP spot in each probably cannot be seen as it is out of the focal plan. Bar 10μm.

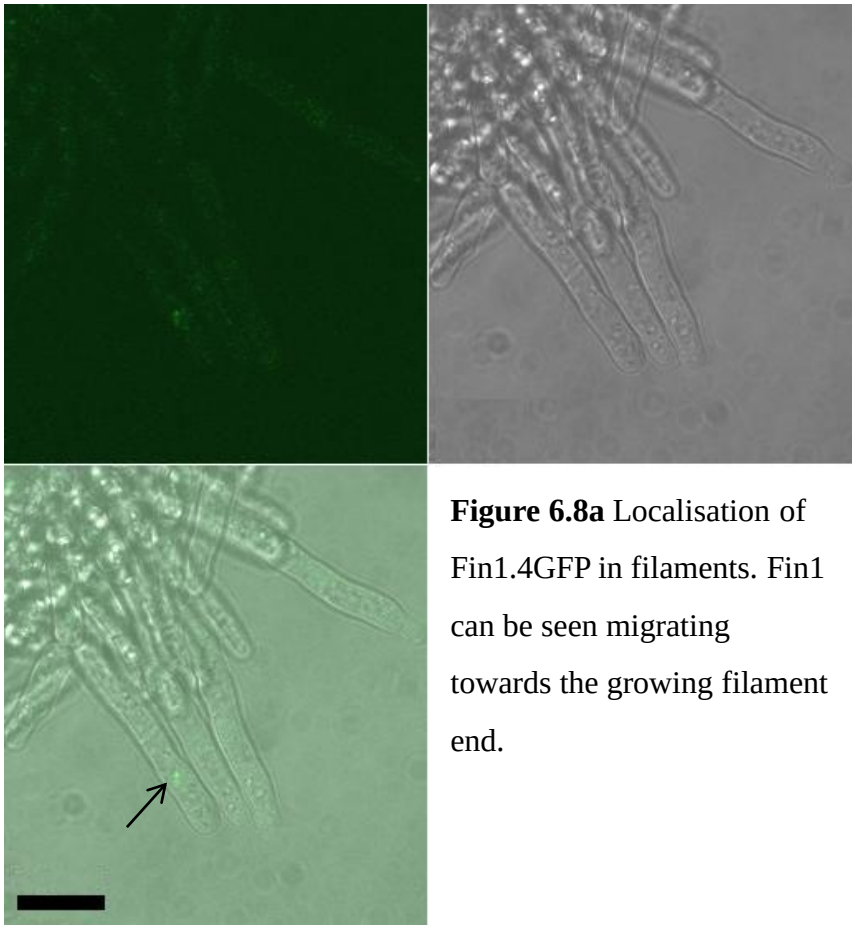


Figure 6.8a Localisation of Fin1.4GFP in filaments. Fin1 can be seen migrating towards the growing filament end.

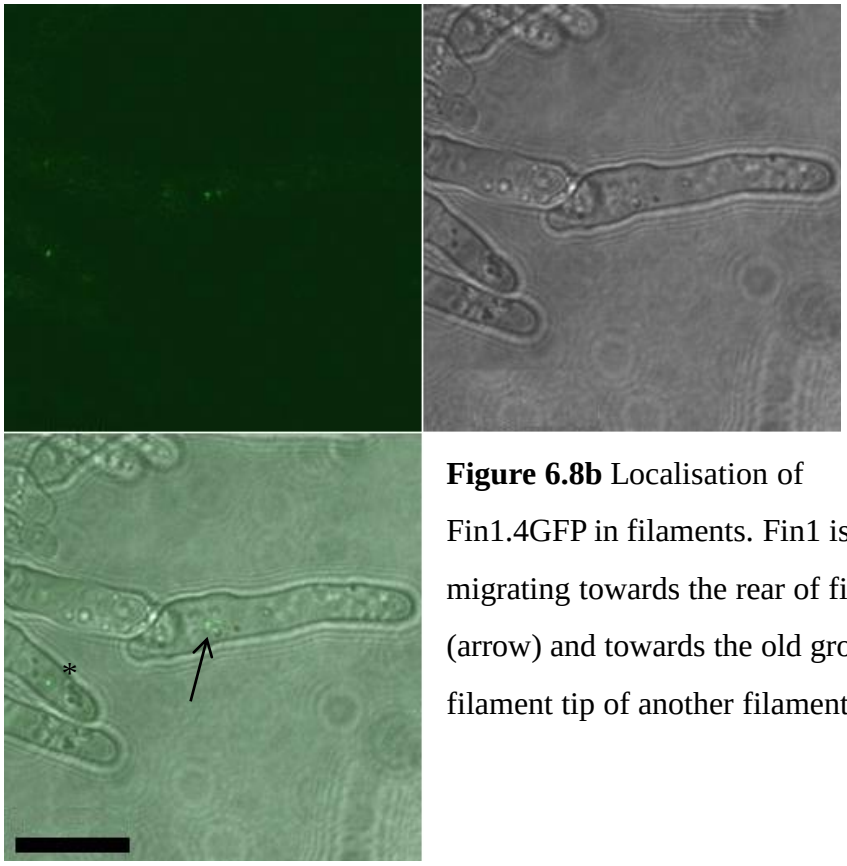


Figure 6.8b Localisation of Fin1.4GFP in filaments. Fin1 is migrating towards the rear of filament (arrow) and towards the old growing filament tip of another filament (*)

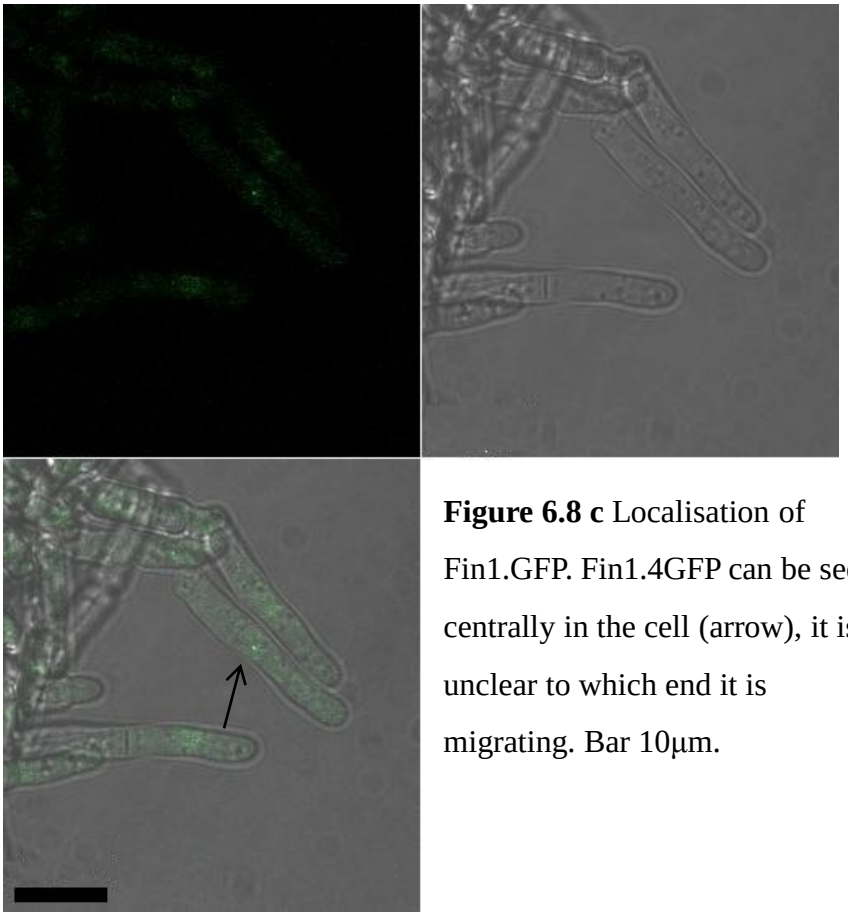


Figure 6.8 c Localisation of Fin1.GFP. Fin1.4GFP can be seen centrally in the cell (arrow), it is unclear to which end it is migrating. Bar 10μm.

actively growing filaments and 19 of these were scored as migrating to the growing filament end. This strongly suggests that during filamentous growth Cdc7.GFP migration is biased towards the growing filament end.

6.3.1iii: Cdc7.GFP is not necessarily a marker for the new SPB in filaments

In single cells Cdc7.GFP is a marker of the new SPB (Grallert et al. 2004). This has been previously determined by the failure of Cdc7.GFP to co-localise with Pcp1.RFP which is a marker of the old SPB (Grallert et al. 2004). This same lack of co-localisation of Pcp1.RFP with Cdc7.GFP has not been established in filaments, therefore it cannot be assumed that Cdc7.GFP is a marker for the new SPB during filamentous growth.

Although the association of Cdc7.GFP with the new SPB cannot yet be confirmed in filaments, analysis of Cdc7.GFP localisation from time lapse microscopy may still be insightful.

6.3.1iv: Two consecutive Cdc7.GFP foci migrated towards the growing filament end

Time lapse microscopy allowed the visualisation of two consecutive Cdc7.GFP foci migrating to the growing cell end. This strongly supports the hypothesis that Cdc7.GFP segregation towards the growing end is biased. The replication time of cells within these filaments was roughly 2.5 hours, therefore the filaments for which only one SPB segregation was noted did not go through two consecutive cell separations within the time-lapse period.

Two filaments exhibited a segregation of Cdc7.GFP to the growing end, followed by a second segregation of Cdc7.GFP to the non-growing end. One of these filaments then appeared slow in growth rate, though the other did not. It may be that Cdc7.GFP migration to the non-growing end prevents further filamentous growth.

The data analysed so far suggests a biased Cdc7.GFP segregation pattern in the lead filament. Is this same bias seen in the secondary cells?

6.3.1v: Cdc7.GFP segregation reverted to an unbiased pattern in secondary cells

Cells that are directly and indirectly behind the leading filament cell are secondary filamentous cells. The growth pattern in these cells is similar to single cells (Dodgson et

al. 2010), so the migration pattern of Cdc7.GFP in these cells was studied to see if the bias that is seen in the leading filament cell remains in the secondary cells. The growing end of a filament is the old end of the cell, the non-growing rear end is the new end of the cell. Therefore in the leading filament Cdc7.GFP exhibits a bias for the old end of the cell. Seven Cdc7.GFP foci were visualised in secondary cells and five of these migrated towards the new end of the cell. Therefore in the secondary cells the bias of Cdc7.GFP to the old end of the cell is lost. The reversion to non-bias segregation pattern in secondary filaments co-insides with reversion to bi-polar growth. This supports the idea that mono-polar growth is associated with the Cdc7.GFP segregation bias. Secondary cells have a growth pattern like single cell yeast and NETO resumes (Dodgson et al 2010). Therefore the cellular changes required for filamentous growth must revert back to the single cell growth pattern. This supports the idea that the lead filament exhibits a unique growth pattern (chapter 5), as the cells behind grow in a similar, if not the same pattern to single cell yeast.

The biased segregation of Cdc7.GFP to the growing end in the leading filament may be a result of one of two events; (1) the biased association of Cdc7.GFP to the new SPB has been lost so Cdc7.GFP now associates with whichever SPB migrates towards the growing filament end or (2) the association of Cdc7.GFP with the new SPB remains and the new SPB has a bias for the growing end.

6.3.1vi: Cdc7.GFP segregation to the growing end may control the site of SIN activation

Cdc7.GFP is a marker for the SPB from where the septation initiation network (SIN) is activated (Sohrmann et al. 1998). Therefore, the constant segregation of Cdc7 to the growing filament end may be to ensure SIN is activated from the growing end of the cell to achieve critical elongation prior to cell division. This elongation may be achieved through a delay of SIN. If SIN were triggered from the non-growing cell end, potentially SIN may be initiated too early and not allow sufficient cell elongation for filamentous growth.

Alternatively, the segregation pattern of Cdc7.GFP may be a result of the new SPB continually migrating to the growing end.

6.3.1vii: Cdc7.GFP segregation to the growing end may control localisation of new SPB

If Cdc7 association to the new SPB still takes place during filamentous growth, then perhaps it is the age of the SPB that is important for filament formation. This may be to ensure the newest formed proteins are in the leading filament cell. In this scenario it may not be important that SIN initiates from the growing filament end but this is a consequence of having the new SPB migrating to the growing filament end. There is still much unknown about the subtle differences between the two SPBs; Grallert *et al*. showed the SPB takes more than one cell cycle to mature, and these changes affect the association of SPB factors (Grallert et al. 2004) so potentially some of these subtle changes may affect filament growth and polarisation.

It is currently unclear which event (age of SPB, SIN initiation or both) is important during filamentous growth. The discussion of processes which may potentially control the segregation of Cdc7.GFP may implicate one event over another.

6.3.1viii: Cdc7.GFP segregation may be controlled by asymmetric protein localisation

To discuss the causes of the biased Cdc7.GFP segregation pattern the difference from the growing and non-growing filament end should first be considered. To date, the only known differences are localisation of two subsets of proteins that favour the growing end and non- growing end. Tea1, Tea4, Pom1 and Mod5 which are all involved in growth polarity, localise at a higher intensity at the non-growing end. Crn1, Bgs4, Cdc42 and Spo2 are all found at higher intensities at the growing end (Dodgson et al. 2010). As protein localisation is the only known differences between the growing and non-growing end could this affect the segregation pattern of Cdc7?

To continuously target the new SPB to the growing filament end the decision for this migration must be made at the very start of SPB segregation when the SPBs are next to each other and central in the cell. Due to the close proximity of the SPBs to one another at this point it would be unlikely that external factors could affect one SPB and not the other, unless there were a structural difference between the two SPBs. A structural difference between the old and new SPB is possible though as yet undefined as SPB duplication in *S. pombe* has not been extensively characterised. Therefore, it would be difficult for the asymmetric protein localisation to affect the SPB which migrates towards the growing end.

Instead of targeting the new SPB to the growing end, either SPB may migrate to the growing end and whichever SPB is in that end recruits Cdc7. In this scenario, the decision of which SPB recruits Cdc7 would not have to be made until later in the cell cycle, once the SPBs have separated enough for differing local factors to affect what is recruited to them. In early anaphase Cdc7 associates with both SPBs; it is only later during anaphase when Spg1 activity is turned off on one SPB that Cdc7 associates at a higher intensity to the other SPB. In single cell growth Spg1 is kept active at the new SPB preferentially (Grallert et al. 2004). If this preference of active Spg1 to the new SPB is somehow turned off, then the SPB that migrates towards the growing end could be kept as the active one by localisation of Spg1 activating protein Etd1. If Etd1localistion is asymmetric to the growing end whichever SPB migrated towards the growing end would be the one that would recruit Cdc7 (figure 6.9). Further work to localise these control proteins would strengthen this hypothesis.
To investigate the involvement of growth polarity determining factors in the segregation of Cdc7.GFP during filamentous growth, Cdc7.GFP was localised in a variety of growth polarity determining deletion strains.

6.3.2: Cdc7.GFP segregation in growth polarity deletion strains is unbiased

Cdc7.GFP localisation data in *tea1Δ, tea2Δ* and *tip1Δ* filamentous cells suggests the bias of Cdc7.GFP segregation has been lost.

6.3.2i: Cell polarity deletion strains exhibited fewer Cdc7.GFP foci

Tea1Δ, tea2Δ and *tip1Δ* deletion strains all exhibited fewer Cdc7.GFP foci, of these, more of them migrated to the non-growing end of the cell in comparison to parental Cdc7.GFP control. This data suggests the segregation bias to the growing filament end has been lost and that the biased segregation of Cdc7 is dependent on the cell polarity machinery. Cdc7.GFP is only visible once it is recruited to the SPB, via Spg1-GTP. The fewer visible foci implies a decrease in Cdc7.GFP association with Spg1-GTP. This could be due to reduced levels of Spg1-GTP or reduced recruitment of Cdc7.GFP to Spg1-GTP. The low number of foci visualised for each deletion strain means the results do not strongly support the conclusion formed and they need to be repeated to confirm this result.

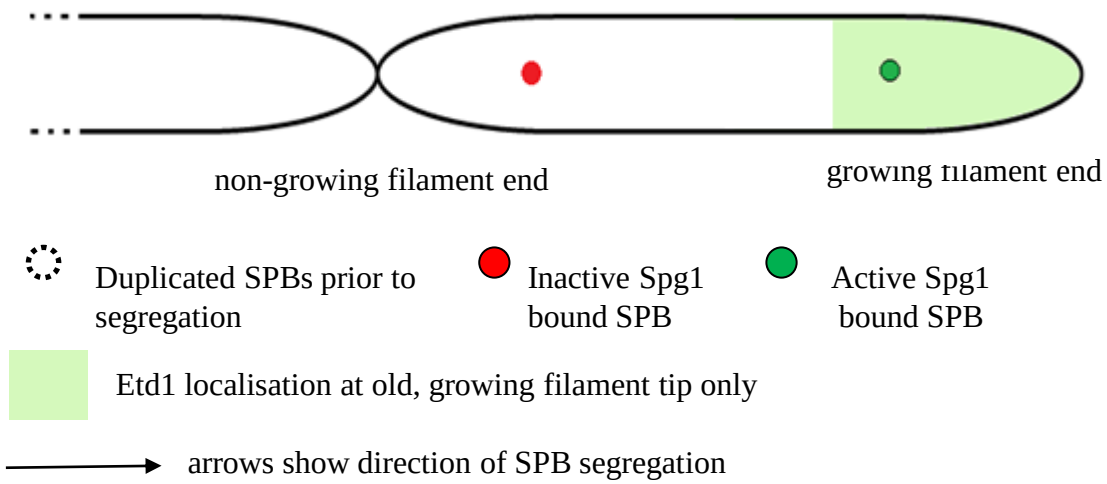


Figure 6.9 Proposed model for Spg1 activation in the growing filament end. As the duplicated SPBs migrate towards opposite filament ends protein localisation could affect activity of proteins on the SPBs. Etd1 is an Spg1 activator therefore localisation to the growing filament end would ensure whichever SPB migrated towards that end would remain Spg1 active (green spot) and recruit Cdc7.

6.3.2ii: Aberrant Etd1 localisation may cause fewer Cdc7.GFP foci in cell polarity deletion strains

The cell polarity mutants may not be able to localise the required components to the growing end, resulting in an inability to maintain Spg1 in its GTP bound state. Therefore fewer Cdc7.GFP foci would be seen in the filaments as there would be no Spg1-GTP for it to be recruited to. Etd1 is the Spg1 activating protein. Asymmetric localisation of Etd1 to the growing filament end would result in consistent activation of Spg1 in the that end. If Etd1 localisation were dependent on the cell polarity determinants then there would be a loss of asymmetry in the representative deletion strains (*tea1Δ, tea2Δ* and *tip1Δ)* resulting in less Spg1 activity and therefore less Cdc7 recruitment (figure 6.10).

Not all cell polarity determining factors display this loss of Cdc7.GFP bias, as shown by analysis of Tea1 and Pom1 cells.

6.3.2iii: Tea4 and Pom1 do not regulate Cdc7.GFP segregation in filamentous growth

To assess whether Pom1 alters Cdc7.GFP segregation pattern, Cdc7.GFP was analysed in *pom1Δ*. The Cdc7.GFP segregation pattern was the same as in the parental Cdc7.GFP strain and implies that Pom1 is not required for biased segregation of Cdc7.GFP. Pom1 is symmetrically localised in single cells but asymmetrically localised in filaments (Dodgson et al. 2010). The lack of effect on Cdc7 segregation implies that the alteration of protein localisation from bi-polar to mono-polar during filamentous growth has multiple consequences and not just an alteration of Cdc7.GFP segregation pattern.

Tea4 is a polarity factor required for the localisation of other cell polarity factors at the cell ends (Tatebe et al. 2005). The *tea4Δ,* A1153 deletion strain exhibits the same pattern of Cdc7.GFP segregation to that of the wild type Cdc7.GFP strain. Tea4 is a scaffold protein for the Tea1 cell end complex which is involved in cell polarity. Tea4 is mainly required for NETO, which does not take place during filamentous growth (Dodgson et al. 2010) therefore the *tea4Δ* deletion strain phenotype is not as severe as *tea1Δ*. *Tea4Δ* does not show the same loss of Cdc7.GFP segregation bias as seen in *tea1Δ*. This suggests Tea4 is not as important in localising the proposed Spg1 activating protein Etd1.

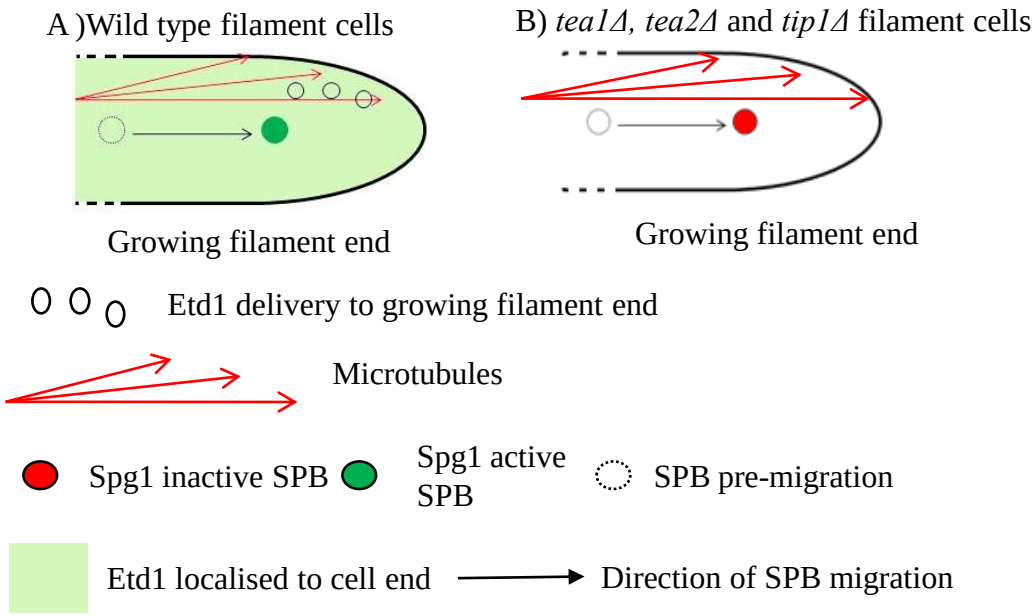


Figure 6.10 Potential model for how Cdc7 recruitment to the SPB may be affected in cell polarity mutants. A) in wild type cells, localisation of potential control factors such as Etd1 to the old growing cell end is facilitated by microtubules. This could cause Spg1 activation on the SPB that migrates to the growing end, thus Cdc7 is always recruited to that SPB. B) In *tea1Δ, tea2Δ* and *tip1Δ* cells, potential control factors such as Etd1 may not be correctly localised, resulting in lack of Spg1-GTP form on the SPB and so lack of Cdc7 recruitment.

6.3.2iv: Fin1 effect on Cdc7.GFP segregation during filamentous growth

Fin1 is a non-essential regulator of SIN (Grallert et al. 2004). To investigate its role in filamentous growth, Cdc7.GFP was expressed in *fin1Δ*. Two Cdc7.GFP foci were seen migrating towards the growing filament end. This suggests Fin1 is not involved in Cdc7 segregation.

6.3.2iva: Cdc7 recruitment to SPB may be independent of Fin1 during filamentous growth

Seven Cdc7.GFP foci were visible in the *fin1Δ* cells analysed, three of these were in the leading filament and four were in the secondary filaments. In each cell that was analysed there was only one visible Cdc7.GFP spot. This is in contrast to single cell data which had previously shown 44% of *fin1Δ* cells exhibited two Cdc7.GFP associated SPBs (Grallert et al. 2004). This suggests the association of Cdc7.GFP to the SPB that migrates towards the growing filament tip is achieved via a separate mechanism to Cdc7 recruitment to the new SPB during single cell growth.

The potential crucial difference between single and filamentous cells in this scenario is the bi- and mono -polar localisation of Etd1 (the Spg1 activating protein) respectively (figure 6.9). In single cells Etd1 localises throughout the cytoplasm during anaphase so would have an equal effect on both SPBs (Garcia-Cortes et al. 2009). Therefore, de-activating factors are required at one SPB to ensure Spg1 is turned off and does not activate SIN. This deactivation is achieved through Cdc16, Byr4 and Fin1 association to the old SPB (Furge et al. 1998; Grallert et al. 2004). If Etd1 exhibits a mono-polar localisation at the growing end during filamentous growth then SIN machinery would not necessarily need to be deactivated at the old SPB in the non-growing end of the cell as there would be no Etd1 activating it. In this scenario Fin1 deletion would have no effect on Cdc7 recruitment to SPBs as the SPB in the non-growing end of the cell would be kept inactive by a lack of Etd1. Single cells do exhibit a bias in localisation of Etd1, but this takes place after cytokinesis and it is used to turn off SIN once the cell has separated to ensure a second SIN is not triggered (Garcia-Cortes et al. 2009). Potentially this mechanism is brought forward during filamentous growth to ensure SIN activation from the growing filament end (figure 6.11). This suggested mechanism could explain the lack of effect of *fin1* deletion on Cdc7.GFP recruitment to the SPB during filamentous growth.

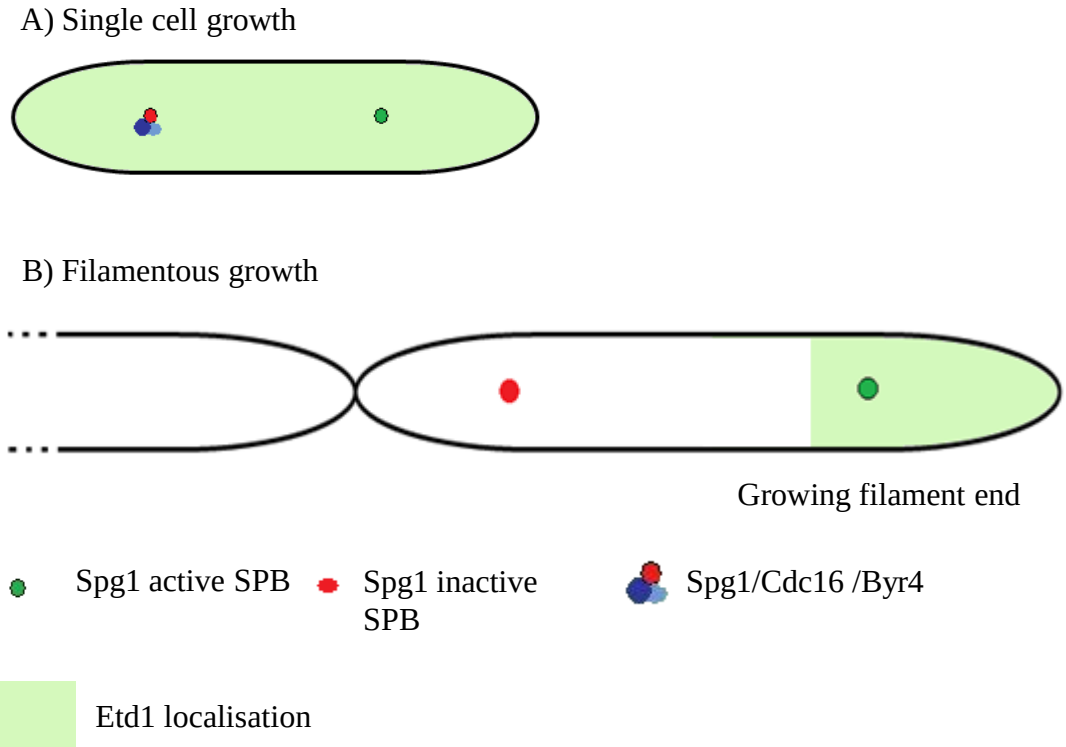


Figure 6.11 Potential filament specific Spg1 activation mechanism. A) In single cells Etd1 is localised throughout the cytoplasm during late anaphase, therefore to ensure SIN is only activated from the new SPB, the old SPB has to be turned off by Spg1 de-activating complex Cdc16/byr4. Potentially during filamentous growth Etd1 localisation is restricted to the growing filament tip, therefore no de-activation of the old SPB is required as there is no Etd1 to activate Spg1 on that SPB.

6.3.2v: Fin1 may regulate filament morphology

Fin1Δ cells appear to have slightly aberrant morphology. *Fin1Δ* was not identified in the screen as exhibiting an aberrant morphology but by crossing the *fin1* deletion into a hyper-filamentous background (A1153), more subtle phenotypes are displayed as the filamentation process is extended. The filaments formed seem to waver in direction more so than the parental A1153 control. This suggest Fin1 may be involved in determining the site of growth during filament formation. The waver in direction is probably due to incorrect positioning of the cell polarity machinery. Fin1 has not been shown to function prior to SPB segregation, so this may be a novel description of Fin1 function.

This concludes the analysis of Cdc7.GFP segregation during filament formation. These results have indicated the importance of SIN during filament formation, therefore analysis of two proteins, Spg1 and Fin1, that are involved in SIN activation may prove insightful to the role of SIN during filament formation.

6.3.3: Localization of Spg1 and Fin1 in filaments

To further analyse the SIN during filament formation, Spg1 and Fin1 were localised in filaments using GFP tagged proteins that were crossed into the highly filamentous A1153 strain. Spg1 is the major controller of SIN and is found on both new and old SPBs (Schmidt et al. 1997).

6.3.3i: Spg1 function in filaments is probably the same as in single cells

If a Spg1.GFP focus is seen near the cell end and the cell is elongated it suggests the SPB has duplicated and migrated towards their relative cell ends *i.e.* there should be two Spg1-GFP foci. If there is a single Spg1-GFP focus localised centrally in the cell and the cell is not elongated it means the SPB has not yet duplicated. In the images presented, only one Spg1 focus is visible in each cell; this is presumably because the SPB has not duplicated, as the SPB is still central to the cell. In some cells Spg1-GFP is visible at one cell end but not the other, most likely because the other end of the cell is in the incorrect focal plane to visualise the fluorescence. These results suggest Spg1function in filaments is the same as in single cells.

6.3.3ii: Fin1 association to the SPB during filamentous growth may be independent of Byr4

Fin1 is a non-essential negative regulator of SIN, which localises to both the old and new SPB in single cells (Grallert et al. 2004). Fin1-GFP can be seen migrating once into the leading growing filament end and once towards the non-growing filament end. In the final image (figure 6.8c) it is unclear to which end it is migrating as it has not yet started to migrate. Fin1 can associates with the SPB via Byr4, a member of the Spg1 GAP complex (Grallert et al. 2004). Figure 6.11 suggests the GAP complex does not associate with the SPBs during filamentous growth. If Fin1 solely associated with the SPB via Byr4 then Fin1 foci would not be visible on SPBs during filamentous growth. However, Fin1 does not exclusively associate to the SPB via Byr4, as in single cells it localises to the new SPB 50% of the time and Byr4 is not found at the new SPB (Grallert et al. 2004). It is still unclear how Fin1 associates with the new SPB, but it seems to associate at a lower intensity than it does to Byr4 (Grallert et al. 2004). This could explain the weakness of signal in these Fin1.4GFP cells. Therefore, during filamentous growth, Fin1 may associate with the old or new SPB via an unknown protein. Additionally, Fin1 does not inhibit SIN exclusively through the GAP complex. Grallert *et al.*, 2004 found that the aberrant phenotype displayed by GAP complex/Fin1 double deletion was more severe than the single GAP complex deletion phenotype. Therefore, Fin1 functions to inhibit SIN by an unknown method that is independent of GAP inhibition. Potentially this attenuated SIN inhibition mechanism which can take place on either old or new SPB, could be in place during filamentous growth to ensure a critical size of cell is reached prior to SIN. This attenuated inhibition could occur at the old or new SPB and so migration to either end, as seen in figure 6.8 would be expected.

6.3.3iii: Fin1 visualisation neither supports or refutes the hypothesis that the new SPB continually migrates towards the growing end

Fin1 can localise to both old and new SPBs in single cells (Grallert et al. 2004), therefore the presence of Fin1 in both growing filament and non-growing filament end neither supports nor refutes the proposal that either SPB migrates toward the growing filament end (section 6.3.2). However, the description of Fin1 association to the SPB as an age dependent process allows further analysis of the Fin1.4GFP localisation pattern within the filaments (Grallert et al. 2004). Fin1 can associate to an old SPB and a new SPB but only if that new SPB has arisen from a duplication of a SPB that was old in the

previous cell division (Grallert et al. 2004) (figure 6.12). If this same age dependent process is true in for Fin1 recruitment to the SPB in filaments as in single cell, it can be used to analyse whether or not the new SPB continuously migrates to the growing end, as discussed in section 6.3.2. If the new SPB continuously migrates to the growing filament end, then Fin1 foci should never be visible at the growing filament end, as it will a) never be an old SPB or b) be duplicated from an old SPB (figure 6.13). Figure 6.8a shows Fin1.4GFP migrating towards the growing filament end. If the Fin1 association process described above is true, the localisation of Fin1.4GFP in the growing filament end suggests this SPB is the old SPB. This supports the proposal that either old or new SPB migrates to the growing end, as discussed in section 6.3.2. However, Fin1 can bind both SPBs after duplication if the SPB prior to duplication was the old SPB from the previous cell division. This could be the case if either old or new SPB migrated toward the growing filament end. Therefore, potentially 50% of the time both SPBs should recruit Fin1 in the leading filament cell. In the four leading filament cells analysed only one Fin1.4GFP SPB was visible. This supports the idea that the new SPB continually migrates to the growing end. However due to the low number of cells analysed this may be a co-incidence. Additionally the lack of recruitment by Byr4 in the proposed model (figure 6.11) may result in decreased association of Fin1.4GFP at the old SPB and the subsequent altered pattern of Fin1 recruitment during filamentous growth. In conclusion, if the proposed model of either SPB migrating towards the growing end is true, then further quantitative analysis of Fin1.4GFP during filamentous growth should show two Fin1 foci in the leading filamentous cell.

6.3.4: Conclusions

Analysis of Cdc7.GFP segregation pattern during filamentous growth has led to a number of conclusions.

1) Cdc7 segregation is biased during filamentous growth. Cdc7 exhibits a preference for the growing filament end.
2) The loss of bias in the secondary cells strengthens the proposal that the leading filament cell exhibits a unique growth phase (chapter 5).
3) Tea1, Tea2 and Tip1 affect the Cdc7 segregation bias.

Additionally, the data presented in this chapter have lead to a number of suggestions about the regulation of Cdc7 segregation bias.

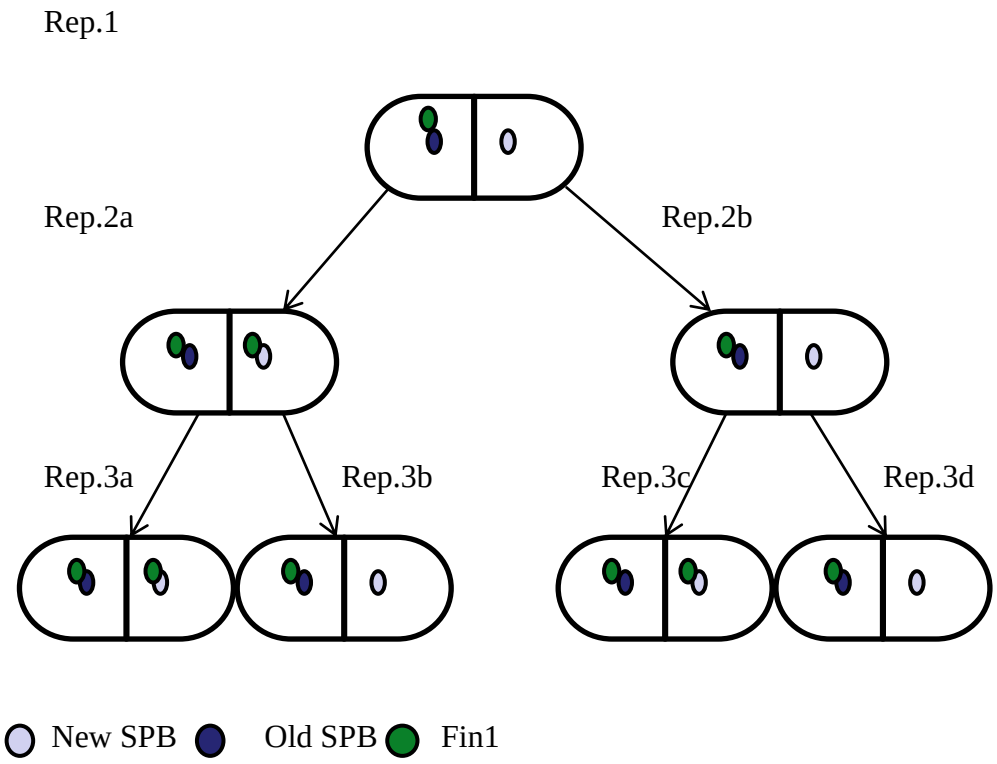


Figure 6.12 Age dependent association of Fin1 to the SPB (Grallert *et al*. 2004). In replication (Rep.) 1 the old SPB was a new SPB in the previous cell cycle, therefore only the old SPB can recruit Fin1. In rep.2a both SPB can recruit Fin1 as the new SPB has been duplicated from an SPB that was the old SPB in the previous cell cycle. In rep.2b only the old SPB can recruit Fin1 as the new SPB has been duplicated from an SPB that was the new SPB in the previous cell cycle. This same philosophy applies for progeny 3a,b,c and d, if the SPB is old then it can recruit Fin1, if it is a new SPB that has been duplicated from an old SPB from the previous cell cycle then it can recruit Fin1, however if it has been duplicated from an SPB that was new in the previous cell cycle it cannot recruit Fin1.

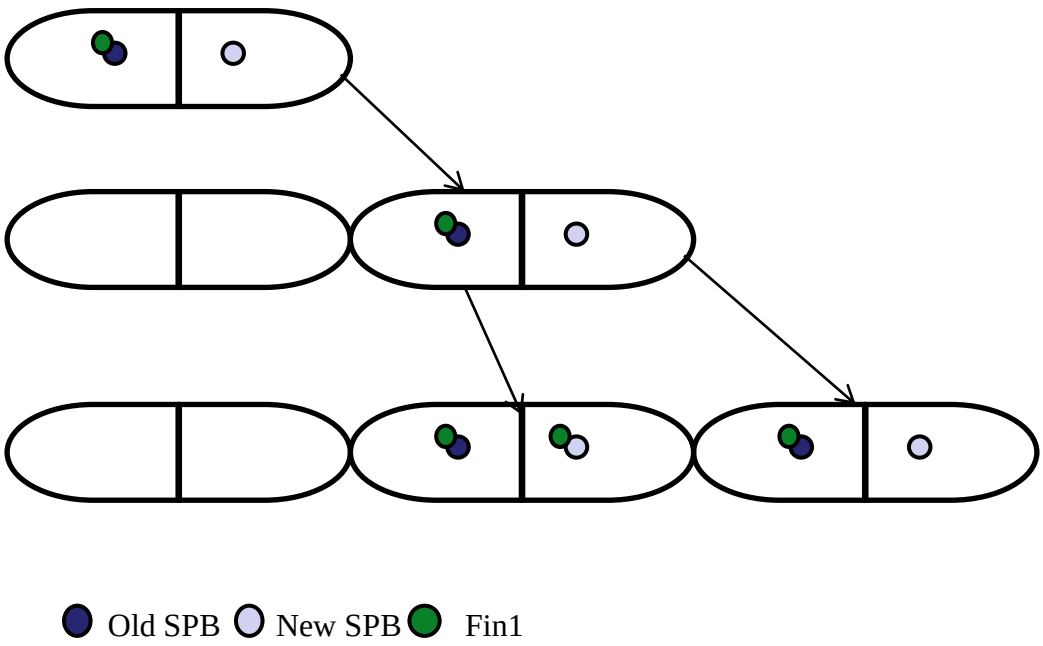


Figure 6.13 Fin1 localisation pattern during filamentous growth if the new SPB continually migrates toward growing tip. If the new SPB always migrates towards the growing tip, then Fin1 should never be visible in this growing tip as the new SPB will always be duplicated from the new SPB of the previous cell division.

1) Etd1 asymmetry in filaments could regulate recruitment of Cdc7 to the SPB in the growing filament end.
2) *Tea1Δ, tea2Δ, tip1Δ* may lose Cdc7 segregation bias as they are unable to asymmetrically localise Etd1.
3) Fin1 may affect polarised growth during filament formation
4) Fin1 recruitment to the SPB during filament formation may be different to the Fin1 recruitment to the SPB in single cells.

There are a number of proposals made in this chapter that require further experimental work to confirm the result. The crucial question that needs to be addressed from this chapter is whether or not the new SPB consistently migrates towards the leading growing end of the filament. Experiments with pcp1.RFP, which localises to the old SPB, could help clarify the history of the SPB in the growing filament end.

Chapter 7: Discussion and future work

7: Invasion is a three stage process

This thesis has described progress in deciphering the changes involved in the transition from single cell to invasive growth. It also presents many further avenues for investigation. The screen identified 177 deletion strains that exhibited an aberrant phenotype. This allowed the description of a tertiary stage of the invasive process: filament formation. This screen utilised deletion strains to investigate the invasive process. This technique is only applicable to non-essential genes. Over-expression of essential genes or construction of specific or conditional mutants may identify roles for these genes during the invasive process.

7.1: An adhesin was identified that may be required for invasive growth

34 strains were identified as non-invasive or poorly-invasive. This included a cell-surface adhesin, Fta5 (Linder et al. 2008), which may be essential for invasion. The only compound that is able to rescue the aberrant invasive phenotype of *fta5Δ* is $FeCl_2$ but iron-stimulated invasion is proposed to by-pass the requirement of normal invasion-associated machinery.

7.1.1: Over-expression of *fta5* to investigate its proposed role as an invasion-associated adhesin

In chapter 3 (section 3.3.3i) Fta5 is implicated as an adhesin required for invasion. If this is true, over-expression of *fta5* should cause a hype-adhesive phenotype. Additionally, some of the non-invasive strains identified in this screen may represent proteins that are responsible for Fta5 expression on the cell surface. Over-expression of Fta5 in the non-invasive strains may allow identification of genes which are involved in its surface localisation as over-expression of Fta5 would rescue the non-invasive phenotype.

7.2: Signalling of cell morphology pathways during invasion

This screen highlighted the importance of the Ras1 morphology-associated signalling as well the Rho1 morphology-associated signalling pathways during the invasive process (Chapter 3, section 3.3.3iv). The involvement of these pathways suggests that multiple signalling pathways are required for invasion. Ras1 signals to Scd1 to activate Cdc42 (Chang et al. 1994). Cdc42 then activates For3 (Martin et al. 2007), which is essential for invasion (Dodgson et al. 2009). This suggests Cdc42 activation is required for invasion.

7.2.1: Over-expression of a constitutively active form of Cdc42 to investigate the role of Ccr1

Scd1 is normally responsible for Cdc42 activation (Papadaki et al. 2002); however, *scd1Δ* did not exhibit an aberrant phenotype in the screen. Cdc42 is an essential protein therefore there must be other activators, perhaps one of these is a novel Cdc42 activating protein required for invasion. *Ccr1Δ,* which is a non-invasive strain, is proposed to encode a protein that associates with Cdc42 during invasive growth (section 3.3.3ii). This proposal is based on the interactions displayed by the *S. cerevisiae* homologue of Ccr1 with *Sc*.CDC42 (Lin et al. 2009). If a similar interaction is responsible for the aberrant phenotype displayed by *ccr1Δ,* expression of a constitutively active form of Cdc42 (Miller et al. 1994) would rescue the aberrant phenotype displayed by *ccr1Δ*.

7.2.2: Investigation into the relationship between Rga8 and Efc25

Rho1 and Ras1 signalling pathways are both proposed to regulate cell morphology (Chapter 3, section 3.3.3iv) but the interactions between these two pathways are unclear. Efc25 is an activating GEF for Ras1(Tratner et al. 1997) and *efc25Δ* is non-invasive. Rga8 is an inhibitory GAP for Rho1 (Yang et al. 2003) and *rga8Δ* is hyper-invasive. Creation of a double deletion strain could determine which of these pathways is dominant during invasive growth.

7.3: The role of cAMP signalling during invasion

This thesis adds further support for the role of cAMP during regulation of the invasion and specifically identifies it as the signalling pathway required for filament formation. The number of strains rescued by growth with a cAMP analogue indicates there are additional signalling pathways involved. Three pathways have been identified as negative regulators of the invasive process; calcineurin, stress-activated MAPK and Rho1-mediated signalling. Calcineurin may be indirectly regulated by cAMP to elicit an invasive response. Additionally it appears Ras1 signalling is required for invasion. Mating, like invasion, is induced by low nitrogen, this screen suggests the mating pathway may be repressed upon stimulation of invasive growth pathway to ensure only one low nitrogen-induced pathway is activated. This suggests, as with many cellular systems, there may be cross regulation amongst these signalling pathways. The rescue effect of iron on some of the non-invasive strains was suggested to be a toxic effect

(Chapter 4). The morphology displayed by the non-filamentous strains in Chapter 5 (section 5.3.4) supports this proposal. All strains, including the 972 control, displayed grossly aberrant morphology which suggested the cells were in a highly toxic environment. The individual effect of the signalling compounds was considered here; perhaps analysis of combination of compounds may highlight further relationships between the pathways.

7.3.1: Combinational investigation of signalling pathways in invasion

Further analysis of the relationships between the cAMP and calcineurin signalling pathways could be achieved through treatment of non-invasive and poorly-invasive strains with a combination of cAMP analogue and FK506.
One of the effects of these signalling pathways is proposed to be asymmetric distribution of proteins which may facilitate invasion (Chapter 3, section 3.3.3iid).

7.3.2: Localisation of Spac4g8.03c.GFP to investigate a suggested asymmetry

In chapter 3 (section 3.3.3iid) Spac4g8.03c, whose deletion prevents invasion, is proposed to be an RNA binding protein (http://old.genedb.org/genedb/), involved with asymmetric localisation of proteins which may be essential for invasive growth. The localisation of a GFP tagged form of Spgac4g8.03c within actively growing filaments may show an asymmetric localisation which would support this idea.

7.4: Regulation of filament formation

The screen identified 138 strains that exhibited aberrations in filamentous growth. These strains represent four novel morphological classes and highlight the complexity of filament formation. Not all these 138 strains exhibit invasion-specific aberrations.

7.4.1: Investigation into the invasion-specific defective strains in class IIb

In chapter 3 (section 3.3.4iva) class IIb strains were described as a heterogeneous mix of strains, some that exhibited an invasion-specific aberrant morphology and some that also showed aberrant morphologies when grown as single cells. A morphological screen of class IIb strains as single cells would identify which genes conferred invasion-specific defects.

7.5: Signalling pathways that regulate filament formation

Regulation of filament formation is thought to involve the same signalling pathways as invasion; cAMP is required for filament formation and calcineurin inhibits it (Chapters 4 and 5). The cAMP effect on the two stages of the invasive process has been shown to be separable and an amplification of cAMP has been suggested in regulation of both stages. The stress-activated MAPK is not essential for the invasive process but it appears to be required to maintain efficient morphology. This role may be regulated by cAMP signalling.

7.5.1: Investigation into the relationship between cAMP and the stress-activated MAPK pathway

In chapter 3 (section 3.3.4iif) the elongated filamentous phenotype displayed by strains grown in the presence of excess cAMP analogue (Prevorovsky et al. 2009) was proposed to be caused by inhibition of the Sty1 stress-activated MAPK pathway. If this is the case, the phenotype displayed by *sty1Δ* should not be exacerbated by addition of excess cAMP analogue to the growth medium. Therefore, addition of excess cAMP analogue (8-Br-cAMP) to the growth medium may strengthen the proposal that cAMP signalling mediates cellular elongation via inhibition of the Sty1 stress-activated signalling pathway.

Additionally, cAMP is proposed to cause elongation during filament formation via delaying mitosis.

7.5.2: Investigation into the relationship between cAMP mediated delay on mitosis and filament formation

Kishimato *et al*. (2000) created a strain (c*dc25Δ, cdc2-w*,+ PN[Cdc2-F15]) that is unable to delay mitosis in response to cAMP. Cdc2-w is a Cdc2 that is independent of Cdc25 and Cdc2-F15 is over-expressed form a plasmid. Cdc2-F15 is un-phosphorylatable on tyrosine 15. These changes create a strain that does not elongate in response to cAMP (Kishimoto et al. 2000). If cellular elongation during filament formation is achieved via a cAMP induced mitotic delay then this strain should not be able to form filaments.

7.5.3: Further investigation into pyridoxal-5-phosphate regulation of filament formation

The role of pyridoxal-5-phosphate in filament formation was investigated in chapter 5 (section 5.3.6), however the results were inconclusive. Spcc18.10 is responsible for production of pyridoxal-5-phosphate from pyridoxal but it has two additional substrates (pyridoxine and pyridoxamine) and two additional products (pyridoxine-5-phosphate and pyridoxamine-5-pohsphate) which can be further metabolised to form pyridoxal-5-phosphate (Morita et al. 2004). Further investigations with these different types of Spcc18.10 substrate and product may confirm or refute if any of these compounds are required during filament formation.

7.6: The role of protein asymmetry in filament formation

The identification of strains that are able to invade but unable to form filamentous protrusions allowed the description of a tertiary stage of the invasive process: filament formation. Filament formation is proposed to be a unique growth phase that involves monopolar extension and cellular elongation. This unique growth phase was proposed to be maintained by a cellular asymmetry.

7.6.1: Localisation of Cyr1.GFP and Git3.GFP in actively growing filaments

Chapter 5 (section 5.3.2iii) suggests asymmetric localisation of Cyr1 and Git3 may contribute to an amplification of cAMP signal in the leading cell of the filament which may be required to maintain monopolar growth and cellular extension. Localisation of Cyr1.GFP and Git3.GFP in actively growing filaments could determine if such an asymmetry occurs in filament formation. If asymmetry does occur, then localising the asymmetric proteins in deletion strains such as *kes1Δ* and *alg10Δ*, which are proposed to regulate such an asymmetry, could confirm or refute this proposal.

7.6.2: Investigation into asymmetric protein localisation during filament formation

Filament formation is associated with a biased Cdc7 segregation pattern (chapter 6). In single cells Cdc7 consistently localises to the new SPB (Grallert et al. 2004), though this has not yet been shown in filaments. Pcp1.RFP is a marker for the old SPB (Grallert et al. 2004). Pcp1.RFP could be expressed in actively growing filaments to determine whether or not the new SPB always migrates towards the growing end of the filament.

Alternatively photo-activatable GFPs like Dendra (Gurskaya et al. 2006) could be used to determine the SPB inheritance pattern in filaments. Photo-activation of a Dendra-GFP tagged SPB core protein such as Pcp1 prior to SPB duplication could enable determination of which SPB migrates towards the growing cell end.
The biased segregation of Cdc7.GFP was proposed to be regulated by asymmetric localisation of a Spg1 activating protein, Etd1 (Chapter 6, section 6.3.2vii). Localisation of Etd1.GFP in actively growing filaments could support or refute this proposal.

7.6.3: Investigation into the relationship between cAMP and Cdc7 segregation

Deletion of growth polarity components Tea1, Tea2, and Tip1 has been shown to affect the segregation pattern of Cdc7.GFP (Chapter 6, section 6.3.4). Additionally, growth polarity components are thought to be effectors of the cAMP signal. This potentially suggests that cAMP may affect the Cdc7.GFP segregation pattern. To investigate this possibility, Cdc7.GFP could be localised in actively growing filaments that had been treated with 8-Br-cAMP. Secondary cells in normal filaments revert to a non-biased Cdc7 segregation pattern and display single cell-like growth (Chapter 6, section 6.3.2v). As secondary cells grown in the presence of 8-Br-cAMP exhibit an elongated morphology that appears identical to the lead filament, Cdc7.GFP may display a biased segregation pattern in the secondary cells as well.

7.7: The role of SIN during filament formation

The segregation bias exhibited by Cdc7.GFP is proposed to ensure the SIN is triggered from the leading filament (Chapter 6, section 6.3.2v). This could be investigated by further analysis of a SIN inhibitor Fin1. Additionally, Ras1 signalling has been shown to interact with Byr4 (Furge et al. 1998), which is a SIN inhibition protein. Ras1, which has been implicated in control of the invasive process, may therefore be involved in regulating the activity of the SIN on the SPBs.

7.7.1: Investigation into Fin1 association to the SPBs

Fin1.GFP has been proposed to exhibit a weaker signal in filaments than single cells (Chapter 6, section 6.3.5ii). This is based on the proposal that Fin1 may associate with the SPB through a unknown factor that does not recruit Fin1 as strongly as Byr4 (Grallert et al. 2004). Quantitative analysis of Fin1.GFP in single cells compared to filaments could determine if the signal emitted is weaker or not.

During filamentous growth Fin1 has been proposed to associate with the SPB in a different pattern to its recruitment in single cells (Chapter 6, section 6.3.5iii). Time lapse microscopy of Fin1.GFP in actively growing filaments could determine the segregation pattern and whether or not it is different to single cells.

7.8: No nitrogen-sensing genes were identified

The aims of this thesis were (a) to learn about the morphology of the invasive process and (b) to learn about the induction of invasion by low nitrogen. Although progress has been made in identifying the mechanisms involved in the regulation of morphology, only one gene (*met14*) was identified that may represent nitrogen sensing apparatus. This may be as there may be a high level of redundancy in the nitrogen sensing machinery. Additionally it may be because this pathway is as yet un-described, *i.e.* some genes that have been identified may be responsible for nitrogen sensing but have not yet been characterised with this function.

The source of nitrogen in the LNB plates is ammonium. The ammonium transmembrane transporter genes (*amt1, amt2* and *amt3*) are only required for invasion at low cell density (unpublished data) (Mitsuzawa 2006). This could imply that other such nitrogen sensing components are only required at low cell density. This screen was carried out at high cell density therefore they would not have been identified.

7.9: Concluding comment

In conclusion, this thesis has identified filament formation as a tertiary stage of the invasive and classified 138 genes which are involved in regulation of this stage. It has highlighted the involvement of Rho1, Ras1, Sty1 and calcineurin mediated signalling during invasion. Additionally it has strengthened the evidence of the role of cAMP. This thesis has shown that cAMP is not only required for invasion but a separate cAMP signal is also required for the tertiary stage of the invasive process, filament formation. This shows that the same signal can control two separate differential stages of development. This may be achieved through integration with other signalling pathways. This thesis also suggests cellular asymmetry, specifically that of the spindle pole body associated proteins, and regulation of the SIN may be a crucial factors in regulation of invasion.

Bibliography

Agarwal, M., K. Papadopoulou, et al. (2010). "Mid1p-dependent regulation of the M-G1 transcription wave in fission yeast." J Cell Sci **123**(Pt 24): 4366-73.

Alonso-Nunez, M. L., H. An, et al. (2005). "Ace2p controls the expression of genes required for cell separation in Schizosaccharomyces pombe." Mol Biol Cell **16**(4): 2003-17.

Amoah-Buahin, E., N. Bone, et al. (2005). "Hyphal Growth in the Fission Yeast Schizosaccharomyces pombe." Eukaryot Cell **4**(7): 1287-97.

An, H., J. L. Morrell, et al. (2004). "Requirements of fission yeast septins for complex formation, localization, and function." Mol Biol Cell **15**(12): 5551-64.

Aoyama, K., H. Aiba, et al. (2001). "Genetic analysis of the His-to-Asp phosphorelay implicated in mitotic cell cycle control: involvement of histidine-kinase genes of Schizosaccharomyces pombe." Biosci Biotechnol Biochem **65**(10): 2347-52.

Arcangioli, B. (1998). "A site- and strand-specific DNA break confers asymmetric switching potential in fission yeast." Embo J **17**(15): 4503-10.

Arcangioli, B. (2000). "Fate of mat1 DNA strands during mating-type switching in fission yeast." EMBO Rep **1**(2): 145-50.

Arellano, M., A. Duran, et al. (1996). "Rho 1 GTPase activates the (1-3)beta-D-glucan synthase and is involved in Schizosaccharomyces pombe morphogenesis." Embo J **15**(17): 4584-91.

Arellano, M., M. H. Valdivieso, et al. (1999). "Schizosaccharomyces pombe protein kinase C homologues, pck1p and pck2p, are targets of rho1p and rho2p and differentially regulate cell integrity." J Cell Sci **112 (Pt 20)**: 3569-78.

Bahler, J. and J. R. Pringle (1998). "Pom1p, a fission yeast protein kinase that provides positional information for both polarized growth and cytokinesis." Genes Dev **12**(9): 1356-70.

Banuett, F. (1998). "Signalling in the yeasts: an informational cascade with links to the filamentous fungi." Microbiol Mol Biol Rev **62**(2): 249-74.

Bathe, M. and F. Chang (2010). "Cytokinesis and the contractile ring in fission yeast: towards a systems-level understanding." Trends Microbiol **18**(1): 38-45.

Batta, G., Z. Szilagyi, et al. (2009). "The involvement of the Schizosaccharomyces pombe sep9/spt8 gene in the regulation of septum cleavage." FEMS Yeast Res **9**(5): 757-67.

Beach, D. H. and A. J. Klar (1984). "Rearrangements of the transposable mating-type cassettes of fission yeast." Embo J **3**(3): 603-10.

Behrens, R. and P. Nurse (2002). "Roles of fission yeast tea1p in the localization of polarity factors and in organizing the microtubular cytoskeleton." J Cell Biol **157**(5): 783-93.

Bensen, E. S., S. G. Filler, et al. (2002). "A forkhead transcription factor is important for true hyphal as well as yeast morphogenesis in Candida albicans." Eukaryot Cell **1**(5): 787-98.

Bertrand, V. and O. Hobert (2009). "Linking asymmetric cell division to the terminal differentiation program of postmitotic neurons in C. elegans." Dev Cell **16**(4): 563-75.

Betschinger, J. and J. A. Knoblich (2004). "Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates." Curr Biol **14**(16): R674-85.

Bharucha, N., J. Ma, et al. (2008). "Analysis of the yeast kinome reveals a network of regulated protein localization during filamentous growth." Mol Biol Cell **19**(7): 2708-17.

Bockmuhl, D. P., S. Krishnamurthy, et al. (2001). "Distinct and redundant roles of the two protein kinase A isoforms Tpk1p and Tpk2p in morphogenesis and growth of Candida albicans." Mol Microbiol **42**(5): 1243-57.

Braun, B. R. and A. D. Johnson (1997). "Control of filament formation in Candida albicans by the transcriptional repressor TUP1." Science **277**(5322): 105-9.

Browning, H., D. D. Hackney, et al. (2003). "Targeted movement of cell end factors in fission yeast." Nat Cell Biol **5**(9): 812-8.

Browning, H., J. Hayles, et al. (2000). "Tea2p is a kinesin-like protein required to generate polarized growth in fission yeast." J Cell Biol **151**(1): 15-28.

Buck, V., J. Quinn, et al. (2001). "Peroxide sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway." Mol Biol Cell **12**(2): 407-19.

Bulmer, R., A. Pic-Taylor, et al. (2004). "The forkhead transcription factor Fkh2 regulates the cell division cycle of Schizosaccharomyces pombe." Eukaryot Cell **3**(4): 944-54.

Burda, P. and M. Aebi (1998). "The ALG10 locus of Saccharomyces cerevisiae encodes the alpha-1,2 glucosyltransferase of the endoplasmic reticulum: the terminal glucose of the lipid-linked oligosaccharide is required for efficient N-linked glycosylation." Glycobiology **8**(5): 455-62.

Busch, K. E. and D. Brunner (2004). "The microtubule plus end-tracking proteins mal3p and tip1p cooperate for cell-end targeting of interphase microtubules." Curr Biol **14**(7): 548-59.

Byrne, S. M. and C. S. Hoffman (1993). "Six git genes encode a glucose-induced adenylate cyclase activation pathway in the fission yeast Schizosaccharomyces pombe." J Cell Sci **105 (Pt 4)**: 1095-100.

Cao, F., S. Lane, et al. (2006). "The Flo8 transcription factor is essential for hyphal development and virulence in Candida albicans." Mol Biol Cell **17**(1): 295-307.

Carpp, L. N., S. G. Shanks, et al. (2007). "Cellular levels of the syntaxin Tlg2p are regulated by a single mode of binding to Vps45p." Biochem Biophys Res Commun **363**(3): 857-60.

Casamayor, A. and M. Snyder (2002). "Bud-site selection and cell polarity in budding yeast." Curr Opin Microbiol **5**(2): 179-86.

Castagnetti, S., R. Behrens, et al. (2005). "End4/Sla2 is involved in establishment of a new growth zone in Schizosaccharomyces pombe." J Cell Sci **118**(Pt 9): 1843-50.

Cavalieri, D., J. P. Townsend, et al. (2000). "Manifold anomalies in gene expression in a vineyard isolate of Saccharomyces cerevisiae revealed by DNA microarray analysis." Proc Natl Acad Sci U S A **97**(22): 12369-74.

Chang, E. C., M. Barr, et al. (1994). "Cooperative interaction of S. pombe proteins required for mating and morphogenesis." Cell **79**(1): 131-41.

Chumnantana, R., K. Hirose, et al. (2001). "Production of pyridoxal phosphate by a mutant strain of Schizosaccharomyces pombe." Biosci Biotechnol Biochem **65**(8): 1789-95.

Cook, J. G., L. Bardwell, et al. (1996). "Two novel targets of the MAP kinase Kss1 are negative regulators of invasive growth in the yeast Saccharomyces cerevisiae." Genes Dev **10**(22): 2831-48.

Cortes, J. C., E. Carnero, et al. (2005). "The novel fission yeast (1,3)beta-D-glucan synthase catalytic subunit Bgs4p is essential during both cytokinesis and polarized growth." J Cell Sci **118**(Pt 1): 157-74.

Dal Santo, P., B. Blanchard, et al. (1996). "The Schizosaccharomyces pombe pyp1 protein tyrosine phosphatase negatively regulates nutrient monitoring pathways." J Cell Sci **109 (Pt 7)**: 1919-25.

Dalgaard, J. Z. and A. J. Klar (1999). "Orientation of DNA replication establishes mating-type switching pattern in S. pombe." Nature **400**(6740): 181-4.

Dalgaard, J. Z. and A. J. Klar (2001). "A DNA replication-arrest site RTS1 regulates imprinting by determining the direction of replication at mat1 in S. pombe." Genes Dev **15**(16): 2060-8.

Datta, A., K. Ganesan, et al. (1989). "Current trends in Candida albicans research." Adv Microb Physiol **30**: 53-88.

de Groot, P. W., Q. Y. Yin, et al. (2007). "Mass spectrometric identification of covalently bound cell wall proteins from the fission yeast Schizosaccharomyces pombe." Yeast **24**(4): 267-78.

Deng, L., R. Sugiura, et al. (2006). "Real-time monitoring of calcineurin activity in living cells: evidence for two distinct Ca2+-dependent pathways in fission yeast." Mol Biol Cell **17**(11): 4790-800.

Dickinson, J. R. (1996). "'Fusel' alcohols induce hyphal-like extensions and pseudohyphal formation in yeasts." Microbiology **142 (Pt 6)**: 1391-7.

Dickinson, J. R. (2008). "Filament formation in Saccharomyces cerevisiae--a review." Folia Microbiol (Praha) **53**(1): 3-14.

Dodgson, J., H. Avula, et al. (2009). "Functional Genomics of Adhesion, Invasion and Mycelial Formation in Schizosaccharomyces pombe." Eukaryot Cell.

Dodgson, J., W. Brown, et al. (2010). "Reorganization of the growth pattern of Schizosaccharomyces pombe in invasive filament formation." Eukaryot Cell **9**(11): 1788-97.

Draskovic, P., A. Saiardi, et al. (2008). "Inositol hexakisphosphate kinase products contain diphosphate and triphosphate groups." Chem Biol **15**(3): 274-86.

Drummond, D. R. and R. A. Cross (2000). "Dynamics of interphase microtubules in Schizosaccharomyces pombe." Curr Biol **10**(13): 766-75.

Dunand-Sauthier, I., C. A. Walker, et al. (2005). "Stress-activated protein kinase pathway functions to support protein synthesis and translational adaptation in response to environmental stress in fission yeast." Eukaryot Cell **4**(11): 1785-93.

Elson, S. L., S. M. Noble, et al. (2009). "An RNA transport system in Candida albicans regulates hyphal morphology and invasive growth." PLoS Genet **5**(9): e1000664.

Enczi, K., M. Yamaguchi, et al. (2007). "Morphology transition genes in the dimorphic fission yeast Schizosaccharomyces japonicus." Antonie Van Leeuwenhoek **92**(2): 143-54.

Feierbach, B. and F. Chang (2001). "Roles of the fission yeast formin for3p in cell polarity, actin cable formation and symmetric cell division." Curr Biol **11**(21): 1656-65.

Feierbach, B., F. Verde, et al. (2004). "Regulation of a formin complex by the microtubule plus end protein tea1p." J Cell Biol **165**(5): 697-707.

Feoktistova, A., D. McCollum, et al. (1999). "Identification and characterization of Schizosaccharomyces pombe asp1(+), a gene that interacts with mutations in the Arp2/3 complex and actin." Genetics **152**(3): 895-908.

Fernandez, F., M. Jannatipour, et al. (1996). "A new stress protein: synthesis of Schizosaccharomyces pombe UDP--Glc:glycoprotein glucosyltransferase mRNA is induced by stress conditions but the enzyme is not essential for cell viability." Embo J **15**(4): 705-13.

Fisher, D. L. and P. Nurse (1996). "A single fission yeast mitotic cyclin B p34cdc2 kinase promotes both S-phase and mitosis in the absence of G1 cyclins." Embo J **15**(4): 850-60.

Fleig, U. N. and K. L. Gould (1991). "Regulation of cdc2 activity in Schizosaccharomyces pombe: the role of phosphorylation." Semin Cell Biol **2**(4): 195-204.

Furge, K. A., K. Wong, et al. (1998). "Byr4 and Cdc16 form a two-component GTPase-activating protein for the Spg1 GTPase that controls septation in fission yeast." Curr Biol **8**(17): 947-54.

Furuya, K. and H. Niki (2010). "The DNA damage checkpoint regulates a transition between yeast and hyphal growth in Schizosaccharomyces japonicus." Mol Cell Biol.

Gancedo, J. M. (2001). "Control of pseudohyphae formation in Saccharomyces cerevisiae." FEMS Microbiol Rev **25**(1): 107-23.

Garcia-Cortes, J. C. and D. McCollum (2009). "Proper timing of cytokinesis is regulated by Schizosaccharomyces pombe Etd1." J Cell Biol **186**(5): 739-53.

Garcia, P., V. Tajadura, et al. (2009). "The Rho1p exchange factor Rgf1p signals upstream from the Pmk1 mitogen-activated protein kinase pathway in fission yeast." Mol Biol Cell **20**(2): 721-31.

Gimeno, C. J., P. O. Ljungdahl, et al. (1992). "Unipolar cell divisions in the yeast S. cerevisiae lead to filamentous growth: regulation by starvation and RAS." Cell **68**(6): 1077-90.

Grallert, A., A. Krapp, et al. (2004). "Recruitment of NIMA kinase shows that maturation of the S. pombe spindle-pole body occurs over consecutive cell cycles and reveals a role for NIMA in modulating SIN activity." Genes Dev **18**(9): 1007-21.

Guertin, D. A., L. Chang, et al. (2000). "The role of the sid1p kinase and cdc14p in regulating the onset of cytokinesis in fission yeast." Embo J **19**(8): 1803-15.

Guirard, B. M. and E. E. Snell (1988). "Physical and kinetic properties of a pyridoxal reductase purified from bakers' yeast." Biofactors **1**(2): 187-92.

Gurskaya, N. G., V. V. Verkhusha, et al. (2006). "Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light." Nat Biotechnol **24**(4): 461-5.

Gutz, H. and F. J. Doe (1973). "Two Different h Mating Types in SCHIZOSACCHAROMYCES POMBE." Genetics **74**(4): 563-9.

Hagan, I. M. (1998). "The fission yeast microtubule cytoskeleton." J Cell Sci **111 (Pt 12)**: 1603-12.

Hayashi, T., M. Hatanaka, et al. (2007). "Rapamycin sensitivity of the Schizosaccharomyces pombe tor2 mutant and organization of two highly phosphorylated TOR complexes by specific and common subunits." Genes Cells **12**(12): 1357-70.

Hayek, P., L. Dib, et al. (2009). "Characterization of Hwp2, a Candida albicans putative GPI-anchored cell wall protein necessary for invasive growth." Microbiol Res.

Hazan, I. and H. Liu (2002). "Hyphal tip-associated localization of Cdc42 is F-actin dependent in Candida albicans." Eukaryot Cell **1**(6): 856-64.

Heitz, M. J., J. Petersen, et al. (2001). "MTOC formation during mitotic exit in fission yeast." J Cell Sci **114**(Pt 24): 4521-32.

Hirayama, S., R. Sugiura, et al. (2003). "Zinc finger protein Prz1 regulates Ca2+ but not Cl- homeostasis in fission yeast. Identification of distinct branches of calcineurin signaling pathway in fission yeast." J Biol Chem **278**(20): 18078-84.

Ho, S., N. Clipstone, et al. (1996). "The mechanism of action of cyclosporin A and FK506." Clin Immunol Immunopathol **80**(3 Pt 2): S40-5.

Hochberg, Y. B. Y. (1995). "Controlling the False Discovery rate: A Practical and Powerful Apprach to Multiple Testing." Journal of the royal stastical society. Series B (Methodological) **57**(1): 289-300.

Hoffman, C. S. (2005). "Glucose sensing via the protein kinase A pathway in Schizosaccharomyces pombe." Biochem Soc Trans **33**(Pt 1): 257-60.

Hoffman, C. S. and F. Winston (1990). "Isolation and characterization of mutants constitutive for expression of the fbp1 gene of Schizosaccharomyces pombe." Genetics **124**(4): 807-16.

Hoffman, C. S. and F. Winston (1991). "Glucose repression of transcription of the Schizosaccharomyces pombe fbp1 gene occurs by a cAMP signaling pathway." Genes Dev **5**(4): 561-71.

Hoffman, M. A. A. a. C. S. (2008). "Schizosaccharomyces pombe Hsp90/Git10 Is Required for

Glucose/cAMP Signaling." GENETICS(178): 1927-1936.

Humphries, C. L., H. I. Balcer, et al. (2002). "Direct regulation of Arp2/3 complex activity and function by the actin binding protein coronin." J Cell Biol **159**(6): 993-1004.

Ike J. Jeon, W. G. I. (1995). "Analyzing food for nutrition labeling and hazardous contaminants."

Ishiguro, J., S. Shimada, et al. (2001). "Characterization of a fission yeast mutant which displays defects in cell wall integrity and cytokinesis." Genes Genet Syst **76**(4): 257-69.

Jiang, W. and R. L. Hallberg (2000). "Isolation and characterization of par1(+) and par2(+): two Schizosaccharomyces pombe genes encoding B' subunits of protein phosphatase 2A." Genetics **154**(3): 1025-38.

Jin, R., C. J. Dobry, et al. (2008). "Large-scale analysis of yeast filamentous growth by systematic gene disruption and overexpression." Mol Biol Cell **19**(1): 284-96.

Johnson, A. E. and K. L. Gould (2011). "Dma1 ubiquitinates the SIN scaffold, Sid4, to impede the mitotic localization of Plo1 kinase." Embo J **30**(2): 341-54.

Kao, R. S., E. Morreale, et al. (2006). "Schizosaccharomyces pombe Git1 is a C2-domain protein required for glucose activation of adenylate cyclase." Genetics **173**(1): 49-61.

Kelleher, D. J., D. Karaoglu, et al. (2001). "Large-scale isolation of dolichol-linked oligosaccharides with homogeneous oligosaccharide structures: determination of steady-state dolichol-linked oligosaccharide compositions." Glycobiology **11**(4): 321-33.

Khademi, S., J. O'Connell, 3rd, et al. (2004). "Mechanism of ammonia transport by Amt/MEP/Rh: structure of AmtB at 1.35 A." Science **305**(5690): 1587-94.

Kim, D. U., J. Hayles, et al. (2010). "Analysis of a genome-wide set of gene deletions in the fission yeast Schizosaccharomyces pombe." Nat Biotechnol **28**(6): 617-23.

Kim, K. H., Y. M. Cho, et al. (2001). "Negative regulation of filamentous growth and flocculation by Lkh1, a fission yeast LAMMER kinase homolog." Biochem Biophys Res Commun **289**(5): 1237-42.

Kishimoto, N. and I. Yamashita (2000). "Cyclic AMP regulates cell size of Schizosaccharomyces pombe through Cdc25 mitotic inducer." Yeast **16**(6): 523-9.

Kitamura, K. and C. Shimoda (1991). "The Schizosaccharomyces pombe mam2 gene encodes a putative pheromone receptor which has a significant homology with the Saccharomyces cerevisiae Ste2 protein." Embo J **10**(12): 3743-51.

Klar, A. J. (2007). "Lessons learned from studies of fission yeast mating-type switching and silencing." Annu Rev Genet **41**: 213-36.

Klar, A. J., M. J. Bonaduce, et al. (1991). "The mechanism of fission yeast mating type interconversion: seal/replicate/cleave model of replication across the double-stranded break site at mat1." Genetics **127**(3): 489-96.

Koprivova, A., M. Suter, et al. (2000). "Regulation of sulfate assimilation by nitrogen in Arabidopsis." Plant Physiol **122**(3): 737-46.

Kozminski, K. G., G. Alfaro, et al. (2006). "Homologues of oxysterol-binding proteins affect Cdc42p- and Rho1p-mediated cell polarization in Saccharomyces cerevisiae." Traffic **7**(9): 1224-42.

Krapp, A., P. Collin, et al. (2008). "Homoeostasis between the GTPase Spg1p and its GAP in the regulation of cytokinesis in S. pombe." J Cell Sci **121**(Pt 5): 601-8.

Krapp, A., S. Schmidt, et al. (2001). "S. pombe cdc11p, together with sid4p, provides an anchor for septation initiation network proteins on the spindle pole body." Curr Biol **11**(20): 1559-68.

Krapp, A. and V. Simanis (2008). "An overview of the fission yeast septation initiation network (SIN)." Biochem Soc Trans **36**(Pt 3): 411-5.

Krogh, B. O. and L. S. Symington (2004). "Recombination proteins in yeast." Annu Rev Genet **38**: 233-71.

Kron, S. J., C. A. Styles, et al. (1994). "Symmetric cell division in pseudohyphae of the yeast Saccharomyces cerevisiae." Mol Biol Cell **5**(9): 1003-22.

Kume, K., T. Koyano, et al. (2011). "Calcineurin ensures a link between the DNA replication checkpoint and microtubule-dependent polarized growth." Nat Cell Biol **13**(3): 234-42.

Kunitomo, H., A. Sugimoto, et al. (1995). "Schizosaccharomyces pombe pac2+ controls the onset of sexual development via a pathway independent of the cAMP cascade." Curr Genet **28**(1): 32-8.

Kuramae, E. E., V. Robert, et al. (2006). "Conflicting phylogenetic position of Schizosaccharomyces pombe." Genomics **88**(4): 387-93.

Labbe, S., B. Pelletier, et al. (2007). "Iron homeostasis in the fission yeast Schizosaccharomyces pombe." Biometals **20**(3-4): 523-37.

Lahoz, A., M. Alcaide-Gavilan, et al. (2010). "Antagonistic roles of PP2A-Pab1 and Etd1 in the control of cytokinesis in fission yeast." Genetics **186**(4): 1261-70.

Landry, S. and C. S. Hoffman (2001). "The git5 Gbeta and git11 Ggamma form an atypical Gbetagamma dimer acting in the fission yeast glucose/cAMP pathway." Genetics **157**(3): 1159-68.

Larsson, O. (1985). "Commitment events in early G1 requirement for the synthesis of dolichol dependent glycoproteins." Cell Biol Int Rep **9**(7): 627-36.

Lejeune, E., M. Bortfeld, et al. (2007). "The chromatin-remodeling factor FACT contributes to centromeric heterochromatin independently of RNAi." Curr Biol **17**(14): 1219-24.

Lengeler, K. B., R. C. Davidson, et al. (2000). "Signal transduction cascades regulating fungal development and virulence." Microbiol Mol Biol Rev **64**(4): 746-85.

Lin, M., K. Grillitsch, et al. (2009). "Modulation of sterol homeostasis by the Cdc42p effectors Cla4p and Ste20p in the yeast Saccharomyces cerevisiae." Febs J **276**(24): 7253-64.

Linder, T. and C. M. Gustafsson (2008). "Molecular phylogenetics of ascomycotal adhesins--a novel family of putative cell-surface adhesive proteins in fission yeasts." Fungal Genet Biol **45**(4): 485-97.

Lindner, P. (1893). "Schizosaccharomyces pombe sp. nov., a new ferment." Wochenschrift fur Braueri.

Liu, H., C. A. Styles, et al. (1993). "Elements of the yeast pheromone response pathway required for filamentous growth of diploids." Science **262**(5140): 1741-4.

Liu, H., C. A. Styles, et al. (1996). "Saccharomyces cerevisiae S288C has a mutation in FLO8, a gene required for filamentous growth." Genetics **144**(3): 967-78.

Liu, X., I. McLeod, et al. (2005). "Molecular analysis of kinetochore architecture in fission yeast." Embo J **24**(16): 2919-30.

Lo, W. S. and A. M. Dranginis (1996). "FLO11, a yeast gene related to the STA genes, encodes a novel cell surface flocculin." J Bacteriol **178**(24): 7144-51.

Loibl, M., G. Grossmann, et al. (2010). "C terminus of Nce102 determines the structure and function of microdomains in the Saccharomyces cerevisiae plasma membrane." Eukaryot Cell **9**(8): 1184-92.

Loiodice, I., J. Staub, et al. (2005). "Ase1p organizes antiparallel microtubule arrays during interphase and mitosis in fission yeast." Mol Biol Cell **16**(4): 1756-68.

Lorenz, M. C. and J. Heitman (1997). "Yeast pseudohyphal growth is regulated by GPA2, a G protein alpha homolog." Embo J **16**(23): 7008-18.

Lorenz, M. C. and J. Heitman (1998). "The MEP2 ammonium permease regulates pseudohyphal differentiation in Saccharomyces cerevisiae." Embo J **17**(5): 1236-47.

Luo, J., Y. Matsuo, et al. (2009). "Phosphatidylethanolamine is required for normal cell morphology and cytokinesis in the fission yeast Schizosaccharomyces pombe." Eukaryot Cell **8**(5): 790-9.

Luo, J., Y. Matsuo, et al. (2009). "Phosphatidylethanolamine Is Required for Normal Cell Morphology and Cytokinesis in the Fission Yeast, Schizosaccharomyces pombe." Eukaryot Cell.

Madhani, H. D. and G. R. Fink (1998). "The control of filamentous differentiation and virulence in fungi." Trends Cell Biol **8**(9): 348-53.

Maere, S., K. Heymans, et al. (2005). "BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks." Bioinformatics **21**(16): 3448-9.

Magidson, V., F. Chang, et al. (2006). "Regulation of cytokinesis by spindle-pole bodies." Nat Cell Biol **8**(8): 891-3.

Malinsky, J., M. Opekarova, et al. (2010). "The lateral compartmentation of the yeast plasma membrane." Yeast **27**(8): 473-8.

Marini, A. M., S. Vissers, et al. (1994). "Cloning and expression of the MEP1 gene encoding an ammonium transporter in Saccharomyces cerevisiae." Embo J **13**(15): 3456-63.

Martin-Castellanos, C., K. Labib, et al. (1996). "B-type cyclins regulate G1 progression in fission yeast in opposition to the p25rum1 cdk inhibitor." Embo J **15**(4): 839-49.

Martin, S. G. (2009). "Microtubule-dependent cell morphogenesis in the fission yeast." Trends Cell Biol **19**(9): 447-54.

Martin, S. G., S. A. Rincon, et al. (2007). "Regulation of the formin for3p by cdc42p and bud6p." Mol Biol Cell **18**(10): 4155-67.

Mata, J. and J. Bahler (2006). "Global roles of Ste11p, cell type, and pheromone in the control of gene expression during early sexual differentiation in fission yeast." Proc Natl Acad Sci U S A **103**(42): 15517-22.

Mata, J. and P. Nurse (1997). "tea1 and the microtubular cytoskeleton are important for generating global spatial order within the fission yeast cell." Cell **89**(6): 939-49.

Matsuyama, A., A. Shirai, et al. (2004). "pDUAL, a multipurpose, multicopy vector capable of chromosomal integration in fission yeast." Yeast **21**(15): 1289-305.

Millar, J. B., C. H. McGowan, et al. (1991). "p80cdc25 mitotic inducer is the tyrosine phosphatase that activates p34cdc2 kinase in fission yeast." Embo J **10**(13): 4301-9.

Miller, P. J. and D. I. Johnson (1994). "Cdc42p GTPase is involved in controlling polarized cell growth in Schizosaccharomyces pombe." Mol Cell Biol **14**(2): 1075-83.

Miseta, A., R. Kellermayer, et al. (1999). "The vacuolar Ca2+/H+ exchanger Vcx1p/Hum1p tightly controls cytosolic Ca2+ levels in S. cerevisiae." FEBS Lett **451**(2): 132-6.

Mishra, M., J. Karagiannis, et al. (2005). "The 14-3-3 protein rad24p modulates function of the cdc14p family phosphatase clp1p/flp1p in fission yeast." Curr Biol **15**(15): 1376-83.

Mitchison, J. M. and P. Nurse (1985). "Growth in cell length in the fission yeast Schizosaccharomyces pombe." J Cell Sci **75**: 357-76.

Mitsuzawa, H. (2006). "Ammonium transporter genes in the fission yeast Schizosaccharomyces pombe: role in ammonium uptake and a morphological transition." Genes Cells **11**(10): 1183-95.

Miwa, T., Y. Takagi, et al. (2004). "Gpr1, a putative G-protein-coupled receptor, regulates morphogenesis and hypha formation in the pathogenic fungus Candida albicans." Eukaryot Cell **3**(4): 919-31.

Mochizuki, N. and M. Yamamoto (1992). "Reduction in the intracellular cAMP level triggers initiation of sexual development in fission yeast." Mol Gen Genet **233**(1-2): 17-24.

Monahan, B. J., J. Villen, et al. (2008). "Fission yeast SWI/SNF and RSC complexes show compositional and functional differences from budding yeast." Nat Struct Mol Biol **15**(8): 873-80.

Mora-Montes, H. M., P. Ponce-Noyola, et al. (2009). "Protein glycosylation in Candida." Future Microbiol **4**(9): 1167-83.

Moreno, S., J. Hayles, et al. (1989). "Regulation of p34cdc2 protein kinase during mitosis." Cell **58**(2): 361-72.

Moreno, S., P. Nurse, et al. (1990). "Regulation of mitosis by cyclic accumulation of p80cdc25 mitotic inducer in fission yeast." Nature **344**(6266): 549-52.

Morita, T., K. Takegawa, et al. (2004). "Disruption of the plr1+ gene encoding pyridoxal reductase of Schizosaccharomyces pombe." J Biochem **135**(2): 225-30.

Mosch, H. U., R. L. Roberts, et al. (1996). "Ras2 signals via the Cdc42/Ste20/mitogen-activated protein kinase module to induce filamentous growth in Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **93**(11): 5352-6.

Moseley, J. B., A. Mayeux, et al. (2009). "A spatial gradient coordinates cell size and mitotic entry in fission yeast." Nature **459**(7248): 857-60.

Moser, B. A. and P. Russell (2000). "Cell cycle regulation in Schizosaccharomyces pombe." Curr Opin Microbiol **3**(6): 631-6.

Mukaiyama, H., M. Nakase, et al. (2010). "Autophagy in the fission yeast Schizosaccharomyces pombe." FEBS Lett **584**(7): 1327-34.
Mulugu, S., W. Bai, et al. (2007). "A conserved family of enzymes that phosphorylate inositol hexakisphosphate." Science **316**(5821): 106-9.
Mutoh, T., K. Nakano, et al. (2005). "Rho1-GEFs Rgf1 and Rgf2 are involved in formation of cell wall and septum, while Rgf3 is involved in cytokinesis in fission yeast." Genes Cells **10**(12): 1189-202.
Nakano, K. and I. Mabuchi (2006). "Actin-capping protein is involved in controlling organization of actin cytoskeleton together with ADF/cofilin, profilin and F-actin crosslinking proteins in fission yeast." Genes Cells **11**(8): 893-905.
Nunez, A., A. Franco, et al. (2010). "Fission yeast receptor of activated C kinase (RACK1) ortholog Cpc2 regulates mitotic commitment through Wee1 kinase." J Biol Chem **285**(53): 41366-73.
Nurse, P. and P. Thuriaux (1980). "Regulatory genes controlling mitosis in the fission yeast Schizosaccharomyces pombe." Genetics **96**(3): 627-37.
Obara, K., T. Sekito, et al. (2006). "Assortment of phosphatidylinositol 3-kinase complexes--Atg14p directs association of complex I to the pre-autophagosomal structure in Saccharomyces cerevisiae." Mol Biol Cell **17**(4): 1527-39.
Obara, T., M. Nakafuku, et al. (1991). "Isolation and characterization of a gene encoding a G-protein alpha subunit from Schizosaccharomyces pombe: involvement in mating and sporulation pathways." Proc Natl Acad Sci U S A **88**(13): 5877-81.
Odds, F. C. (1985). "Morphogenesis in Candida albicans." Crit Rev Microbiol **12**(1): 45-93.
Okazaki, N., K. Okazaki, et al. (1998). "Novel factor highly conserved among eukaryotes controls sexual development in fission yeast." Mol Cell Biol **18**(2): 887-95.
Onishi, M., M. Iida, et al. (2007). "Schizosaccharomyces pombe Sst4p, a conserved Vps27/Hrs homolog, functions downstream of phosphatidylinositol 3-kinase Pik3p to mediate proper spore formation." Eukaryot Cell **6**(12): 2343-53.
Papadaki, P., V. Pizon, et al. (2002). "Two ras pathways in fission yeast are differentially regulated by two ras guanine nucleotide exchange factors." Mol Cell Biol **22**(13): 4598-606.
Parker, L. L., S. A. Walter, et al. (1993). "Phosphorylation and inactivation of the mitotic inhibitor Wee1 by the nim1/cdr1 kinase." Nature **363**(6431): 736-8.
Pelletier, B., J. Beaudoin, et al. (2002). "Fep1, an iron sensor regulating iron transporter gene expression in Schizosaccharomyces pombe." J Biol Chem **277**(25): 22950-8.
Pereira, G. and E. Schiebel (2001). "The role of the yeast spindle pole body and the mammalian centrosome in regulating late mitotic events." Curr Opin Cell Biol **13**(6): 762-9.
Pereira, G., T. U. Tanaka, et al. (2001). "Modes of spindle pole body inheritance and segregation of the Bfa1p-Bub2p checkpoint protein complex." Embo J **20**(22): 6359-70.
Petit, C. S., S. Mehta, et al. (2005). "Ace2p contributes to fission yeast septin ring assembly by regulating mid2+ expression." J Cell Sci **118**(Pt 24): 5731-42.
Philip, B. and D. E. Levin (2001). "Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1." Mol Cell Biol **21**(1): 271-80.

Pohlmann, J. and U. Fleig (2010). "Asp1, a conserved 1/3 inositol polyphosphate kinase, regulates the dimorphic switch in Schizosaccharomyces pombe." Mol Cell Biol **30**(18): 4535-47.

Prevorovsky, M., J. Stanurova, et al. (2009). "High environmental iron concentrations stimulate adhesion and invasive growth of Schizosaccharomyces pombe." FEMS Microbiol Lett.

Pruyne, D. and A. Bretscher (2000). "Polarization of cell growth in yeast." J Cell Sci **113 (Pt 4)**: 571-85.

Rajavel, M., B. Philip, et al. (1999). "Mid2 is a putative sensor for cell integrity signaling in Saccharomyces cerevisiae." Mol Cell Biol **19**(6): 3969-76.

Reiter, W., S. Watt, et al. (2008). "Fission yeast MAP kinase Sty1 is recruited to stress-induced genes." J Biol Chem **283**(15): 9945-56.

Rhind, N., Z. Chen, et al. (2011). "Comparative functional genomics of the fission yeasts." Science **332**(6032): 930-6.

Ribar, B., A. Grallert, et al. (1999). "Deletion of the sep1(+) forkhead transcription factor homologue is not lethal but causes hyphal growth in Schizosaccharomyces pombe." Biochem Biophys Res Commun **263**(2): 465-74.

Ribas, J. C., M. Diaz, et al. (1991). "Isolation and characterization of Schizosaccharomyces pombe mutants defective in cell wall (1-3)beta-D-glucan." J Bacteriol **173**(11): 3456-62.

Roberts, R. L. and G. R. Fink (1994). "Elements of a single MAP kinase cascade in Saccharomyces cerevisiae mediate two developmental programs in the same cell type: mating and invasive growth." Genes Dev **8**(24): 2974-85.

Rodriguez-Navarro, S., B. Llorente, et al. (2002). "Functional analysis of yeast gene families involved in metabolism of vitamins B1 and B6." Yeast **19**(14): 1261-76.

Rupp, S., E. Summers, et al. (1999). "MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast FLO11 gene." Embo J **18**(5): 1257-69.

Russell, P. and P. Nurse (1987). "Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog." Cell **49**(4): 559-67.

Rustici, G., H. van Bakel, et al. (2007). "Global transcriptional responses of fission and budding yeast to changes in copper and iron levels: a comparative study." Genome Biol **8**(5): R73.

Rutherford, K., J. Parkhill, et al. (2000). "Artemis: sequence visualization and annotation." Bioinformatics **16**(10): 944-5.

Samejima, I., P. C. Lourenco, et al. (2005). "Fission yeast mto2p regulates microtubule nucleation by the centrosomin-related protein mto1p." Mol Biol Cell **16**(6): 3040-51.

Samejima, I., S. Mackie, et al. (1997). "Multiple modes of activation of the stress-responsive MAP kinase pathway in fission yeast." Embo J **16**(20): 6162-70.

Schmidt, A., J. Kunz, et al. (1996). "TOR2 is required for organization of the actin cytoskeleton in yeast." Proc Natl Acad Sci U S A **93**(24): 13780-5.

Schmidt, S., M. Sohrmann, et al. (1997). "The Spg1p GTPase is an essential, dosage-dependent inducer of septum formation in Schizosaccharomyces pombe." Genes Dev **11**(12): 1519-34.

Shannon, P., A. Markiel, et al. (2003). "Cytoscape: a software environment for integrated models of biomolecular interaction networks." Genome Res **13**(11): 2498-504.

Shen, J., L. E. Cowen, et al. (2008). "The Candida albicans pescadillo homolog is required for normal hypha-to-yeast morphogenesis and yeast proliferation." Proc Natl Acad Sci U S A **105**(52): 20918-23.

Sheu, Y. J., B. Santos, et al. (1998). "Spa2p interacts with cell polarity proteins and signaling components involved in yeast cell morphogenesis." Mol Cell Biol **18**(7): 4053-69.

Shimoda, C. (2004). "Forespore membrane assembly in yeast: coordinating SPBs and membrane trafficking." J Cell Sci **117**(Pt 3): 389-96.

Shiozaki, K. and P. Russell (1995). "Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast." Nature **378**(6558): 739-43.

Shor, B., J. Calaycay, et al. (2003). "Cpc2/RACK1 is a ribosome-associated protein that promotes efficient translation in Schizosaccharomyces pombe." J Biol Chem **278**(49): 49119-28.

Sio, S. O., T. Suehiro, et al. (2005). "The role of the regulatory subunit of fission yeast calcineurin for in vivo activity and its relevance to FK506 sensitivity." J Biol Chem **280**(13): 12231-8.

Sipiczki, M., B. Grallert, et al. (1993). "Mycelial and syncytial growth in Schizosaccharomyces pombe induced by novel septation mutations." J Cell Sci **104 (Pt 2)**: 485-93.

Sipiczki, M., K. Takeo, et al. (1998). "Growth polarity transitions in a dimorphic fission yeast." Microbiology **144 (Pt 12)**: 3475-85.

Sipiczki, M., K. Takeo, et al. (1998). "Environmentally controlled dimorphic cycle in a fission yeast." Microbiology **144 (Pt 5)**: 1319-30.

Sladewski, T. E., M. J. Previs, et al. (2009). "Regulation of fission yeast myosin-II function and contractile ring dynamics by regulatory light-chain and heavy-chain phosphorylation." Mol Biol Cell **20**(17): 3941-52.

Snaith, H. A., I. Samejima, et al. (2005). "Multistep and multimode cortical anchoring of tea1p at cell tips in fission yeast." Embo J **24**(21): 3690-9.

Snaith, H. A. and K. E. Sawin (2003). "Fission yeast mod5p regulates polarized growth through anchoring of tea1p at cell tips." Nature **423**(6940): 647-51.

Sohrmann, M., S. Schmidt, et al. (1998). "Asymmetric segregation on spindle poles of the Schizosaccharomyces pombe septum-inducing protein kinase Cdc7p." Genes Dev **12**(1): 84-94.

Soto, T., A. Nunez, et al. (2007). "Transduction of centrifugation-induced gravity forces through mitogen-activated protein kinase pathways in the fission yeast Schizosaccharomyces pombe." Microbiology **153**(Pt 5): 1519-29.

Stajich, J. E., M. L. Berbee, et al. (2009). "The fungi." Curr Biol **19**(18): R840-5.

Stathopoulos, A. M. and M. S. Cyert (1997). "Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast." Genes Dev **11**(24): 3432-44.

Ste´ phanie La Carbona1, C. L. G. a. X. L. G. (2006). "Fission yeast skeleton and cell polarity factors; connecting at the cortex." Biology of the Cell **Volume 98** (11): 619-631.

Stettler, S., E. Warbrick, et al. (1996). "The wis1 signal transduction pathway is required for expression of cAMP-repressed genes in fission yeast." J Cell Sci **109 (Pt 7)**: 1927-35.

Stolz, J., H. J. Wohrmann, et al. (2005). "Amiloride uptake and toxicity in fission yeast are caused by the pyridoxine transporter encoded by bsu1+ (car1+)." Eukaryot Cell **4**(2): 319-26.

Styrkarsdottir, U., R. Egel, et al. (1993). "The smt-0 mutation which abolishes mating-type switching in fission yeast is a deletion." Curr Genet **23**(2): 184-6.
Sudbery, P., N. Gow, et al. (2004). "The distinct morphogenic states of Candida albicans." Trends Microbiol **12**(7): 317-24.
Sugimoto, A., Y. Iino, et al. (1991). "Schizosaccharomyces pombe ste11+ encodes a transcription factor with an HMG motif that is a critical regulator of sexual development." Genes Dev **5**(11): 1990-9.
Sugiura, R., A. Kita, et al. (2003). "Feedback regulation of MAPK signalling by an RNA-binding protein." Nature **424**(6951): 961-5.
Sugiura, R., T. Toda, et al. (1999). "The MAPK kinase Pek1 acts as a phosphorylation-dependent molecular switch." Nature **399**(6735): 479-83.
Sugiura, R., T. Toda, et al. (1998). "pmp1+, a suppressor of calcineurin deficiency, encodes a novel MAP kinase phosphatase in fission yeast." Embo J **17**(1): 140-8.
Svoboda, K., C. F. Schmidt, et al. (1993). "Direct observation of kinesin stepping by optical trapping interferometry." Nature **365**(6448): 721-7.
Tanaka, K., J. Davey, et al. (1993). "Schizosaccharomyces pombe map3+ encodes the putative M-factor receptor." Mol Cell Biol **13**(1): 80-8.
Tanaka, K., J. Petersen, et al. (2001). "The role of Plo1 kinase in mitotic commitment and septation in Schizosaccharomyces pombe." Embo J **20**(6): 1259-70.
Tanaka, N., Y. Fujita, et al. (2005). "Characterization of O-mannosyltransferase family in Schizosaccharomyces pombe." Biochem Biophys Res Commun **330**(3): 813-20.
Tatebe, H., K. Nakano, et al. (2008). "Pom1 DYRK regulates localization of the Rga4 GAP to ensure bipolar activation of Cdc42 in fission yeast." Curr Biol **18**(5): 322-30.
Tatebe, H., K. Shimada, et al. (2005). "Wsh3/Tea4 is a novel cell-end factor essential for bipolar distribution of Tea1 and protects cell polarity under environmental stress in S. pombe." Curr Biol **15**(11): 1006-15.
Thevelein, J. M., R. Gelade, et al. (2005). "Nutrient sensing systems for rapid activation of the protein kinase A pathway in yeast." Biochem Soc Trans **33**(Pt 1): 253-6.
Thomson, A. M., J. T. Rogers, et al. (1999). "Iron-regulatory proteins, iron-responsive elements and ferritin mRNA translation." Int J Biochem Cell Biol **31**(10): 1139-52.
Toda, T., S. Cameron, et al. (1987). "Three different genes in S. cerevisiae encode the catalytic subunits of the cAMP-dependent protein kinase." Cell **50**(2): 277-87.
Tomlin, G. C., J. L. Morrell, et al. (2002). "The spindle pole body protein Cdc11p links Sid4p to the fission yeast septation initiation network." Mol Biol Cell **13**(4): 1203-14.
Toney, M. D. (2005). "Reaction specificity in pyridoxal phosphate enzymes." Arch Biochem Biophys **433**(1): 279-87.
Tran, P. T., L. Marsh, et al. (2001). "A mechanism for nuclear positioning in fission yeast based on microtubule pushing." J Cell Biol **153**(2): 397-411.
Tratner, I., A. Fourticq-Esqueoute, et al. (1997). "Cloning and characterization of the S. pombe gene efc25+, a new putative guanine nucleotide exchange factor." Gene **193**(2): 203-10.
Umeda, M., S. Izaddoost, et al. (2005). "The fission yeast Schizosaccharomyces pombe has two importin-alpha proteins, Imp1p and Cut15p, which have common and unique functions in nucleocytoplasmic transport and cell cycle progression." Genetics **171**(1): 7-21.

Uzawa, S., F. Li, et al. (2004). "Spindle pole body duplication in fission yeast occurs at the G1/S boundary but maturation is blocked until exit from S by an event downstream of cdc10+." Mol Biol Cell **15**(12): 5219-30.

van Aelst, L., A. W. Jans, et al. (1991). "Involvement of the CDC25 gene product in the signal transmission pathway of the glucose-induced RAS-mediated cAMP signal in the yeast Saccharomyces cerevisiae." J Gen Microbiol **137**(2): 341-9.

van Drogen, F. and M. Peter (2002). "Spa2p functions as a scaffold-like protein to recruit the Mpk1p MAP kinase module to sites of polarized growth." Curr Biol **12**(19): 1698-703.

Vassarotti, A. and J. D. Friesen (1985). "Isolation of the fructose-1,6-bisphosphatase gene of the yeast Schizosaccharomyces pombe. Evidence for transcriptional regulation." J Biol Chem **260**(10): 6348-53.

Vinod, P. K. and K. V. Venkatesh (2007). "Specificity of MAPK signaling towards FLO11 expression is established by crosstalk from cAMP pathway." Syst Synth Biol **1**(2): 99-108.

Wang, C. L. and D. J. Oliver (1997). "Glutathione synthetase: similarities of the proteins from Schizosaccharomyces pombe and Arabidopsis thaliana." Biochem J **326 (Pt 2)**: 563-6.

Wang, H. and D. Vavylonis (2008). "Model of For3p-mediated actin cable assembly in fission yeast." PLoS One **3**(12): e4078.

Welton, R. M. and C. S. Hoffman (2000). "Glucose monitoring in fission yeast via the Gpa2 galpha, the git5 Gbeta and the git3 putative glucose receptor." Genetics **156**(2): 513-21.

White, M. G., S. Piccirillo, et al. (2011). "Flo11p adhesin required for meiotic differentiation in Saccharomyces cerevisiae minicolonies grown on plastic surfaces." FEMS Yeast Res **11**(2): 223-32.

Whiteway, M. and C. Bachewich (2007). "Morphogenesis in Candida albicans." Annu Rev Microbiol **61**: 529-53.

Willer, T., M. Brandl, et al. (2005). "Protein O-mannosylation is crucial for cell wall integrity, septation and viability in fission yeast." Mol Microbiol **57**(1): 156-70.

Won, M., Y. J. Jang, et al. (2001). "Pleckstrin homology domain interacts with Rkp1/Cpc2, a RACK1 homolog, to modulate Pck2-mediated signaling process in Schizosaccharomyces pombe." Biochem Biophys Res Commun **289**(5): 987-92.

Won, M., S. K. Park, et al. (2001). "Rkp1/Cpc2, a fission yeast RACK1 homolog, is involved in actin cytoskeleton organization through protein kinase C, Pck2, signaling." Biochem Biophys Res Commun **282**(1): 10-5.

Wood, V., R. Gwilliam, et al. (2002). "The genome sequence of Schizosaccharomyces pombe." Nature **415**(6874): 871-80.

Wu, J. Q., Y. Ye, et al. (2010). "Cooperation between the septins and the actomyosin ring and role of a cell-integrity pathway during cell division in fission yeast." Genetics **186**(3): 897-915.

Xiang, Z., V. Wood, et al. (2000). "The mating-type region of Schizosaccharomyces pombe h(-S) 972: sequencing and analysis of 69 kb including the expressed mat1 locus." Yeast **16**(11): 1061-7.

Xu, H. P., M. White, et al. (1994). "Concerted action of RAS and G proteins in the sexual response pathways of Schizosaccharomyces pombe." Mol Cell Biol **14**(1): 50-8.

Xue-Franzen, Y., S. Kjaerulff, et al. (2006). "Genomewide identification of pheromone-targeted transcription in fission yeast." BMC Genomics **7**: 303.

Yang, P., Y. Qyang, et al. (2003). "The novel Rho GTPase-activating protein family protein, Rga8, provides a potential link between Cdc42/p21-activated kinase and Rho signaling pathways in the fission yeast, Schizosaccharomyces pombe." J Biol Chem **278**(49): 48821-30.

Yang, Y., H. C. Tsui, et al. (1998). "Identification and function of the pdxY gene, which encodes a novel pyridoxal kinase involved in the salvage pathway of pyridoxal 5'-phosphate biosynthesis in Escherichia coli K-12." J Bacteriol **180**(7): 1814-21.

Yoshida, T., T. Toda, et al. (1994). "A calcineurin-like gene ppb1+ in fission yeast: mutant defects in cytokinesis, cell polarity, mating and spindle pole body positioning." J Cell Sci **107 (Pt 7)**: 1725-35.

Zaitsevskaya-Carter, T. and J. A. Cooper (1997). "Spm1, a stress-activated MAP kinase that regulates morphogenesis in S.pombe." Embo J **16**(6): 1318-31.

Zupan, J. and P. Raspor (2008). "Quantitative agar-invasion assay." J Microbiol Methods **73**(2): 100-4.

Websites

http://www.melford.co.uk/, date last accessed 12/08/2011

http://www.fisher.co.uk/, date last accessed 12/08/2011

http://www.formedium.com/uk, date last accessed 12/08/2011

http://www.invitrogen.com, date last accessed 12/08/2011

http://www.biometra.de, date last accessed 12/08/2011

http://www.biotium.com, date last accessed 12/08/2011

http://www.qiagen.com, date last accessed 12/08/2011

http://www.glass-bottom-dishes.com/, date last accessed 12/08/2011

http://www.photoshop.com/, date last accessed 12/08/2011

http://www.fao.org/, date last accessed 12/08/2011

http://www.riken.jp/SPD/36/36H03.htm, date last accessed 12/08/2011

http://www.yeastgenome.or, date last accessed 12/08/2011

http://pombe.kaist.ac.kr/nbtsupp/protocols/gen_anal_haploid.php, date last accessed 12/08/2011

http://old.genedb.org/genedb/pombe, date last accessed 12/08/2011

http://pombe.bioneer.co.kr/, date last accessed 12/08/2011

http://office.microsoft.com/en-gb/access/, date last accessed 12/08/2011

http://rsbweb.nih.gov/ij/index.html, date last accessed 12/08/2011

http://dna.brc.riken.jp/, date last accessed 12/08/2011

http://old.genedb.org/genedb/pombe/, date last accessed 12/08/2011

N	X	corrected p value	Description	n	x	Genes
5230	18	1.74E-02	cell adhesion	16	2	SPAC1F8.06 SPAC22A12.07C
5230	18	2.07E-03	positive regulation of specific transcription from RNA polymerase II promoter	19	3	SPBC1289.10C SPAC2F7.08C SPCC1494.10
5230	18	3.52E-02	chromatin remodelling	206	4	SPBC21C3.02C SPAC2F7.08C SPBC30B4.04C SPBC609.05

Appendix 3.1 BiNGO data set for non-invasive strains analysis. N; total number of genes in reference gene set X; total number of genes in group to be analysed for over representation n; number of genes annotated with GO term in reference gene set x; number of genes annotated with GO term in group of genes under analysis

N	X	corrected p value	Description	n	x	Genes
5185	16	1.16E-02	histone methylation	19	2	SPAC664.03 SPBC18H10.06C
5230	16	1.48E-04	negative regulation of transcription by glucose	11	3	SPBC32H8.07 SPAC23H3.13C SPAC26F1.10C
5230	16	7.23E-04	regulation of transcription	351	7	SPAC32A11.03C SPBC32H8.07 SPBC530.08 SPAC23H3.13C SPAC26F1.10C SPAC23E2.01 SPCC1223.13
5230	16	2.0917E-4	Response to external stimulus	86	5	SPBC15D4.07C SPBC32H8.07 SPAC23H3.13C SPAC26F1.10C SPAC23E2.01
5230	16	7.7002E-3	cAMP mediated signalling	13	2	SPBC32H8.07 SPAC23H3.13C

Appendix 3.2 BiNGO data set for poorly-invasive strains analysis. N; total number of genes in reference gene set X; total number of genes in group to be analysed for over representation n; number of genes annotated with GO term in reference gene set x; number of genes annotated with GO term in group of genes under analysis

Gene ID	Gene Name	Gene Description
SPBC1105.10	*rav1*	Involved in cellular response to calcium ion
SPAC9G1.12	*cpd1*	tRNA methyltransferase
SPCC4G3.15c	*not2*	involved in regulation of transcription
SPAC16C9.06c	*upf1*	RNA dependent ATP helicase, involved in mRNA catabolism
SPAC23A1.07		ubiquitin-protein ligase (E3) (predicted)
SPAC821.05	*eIF3h*	eukaryotic translation initiation factor eIF3h
SPBC2F12.11c	*rep2*	transcription factor, DSC1/MBF transcription factor complex,
SPAC1687.22c	*puf3*	RNA-binding protein, involved in cellular response to stress
SPCC1223.05c	*rpl3702*	60S ribosomal protein L37
SPCC613.06	*rpl902*	60S ribosomal protein L9
SPCC663.04	*rpl39*	60S ribosomal protein L39
SPBC17G9.07	*rps2402*	40S ribosomal protein S24
SPAC3H8.10	*spo20*	involved in sporulation (required)
SPAC29A4.16	*hal4*	serine/threonine protein kinase (predicted)
SPAC17A5.04c	*mde10*	metallo peptidase, involved in sporulation
SPBC9B6.07	*nop52*	involved in rRNA processing (predicted)
SPAC13A11.05		cytosol aminopeptidase, peptidase family M17
SPBC16C6.11	*rpl3201*	60S ribosomal protein L32
SPAC3A12.10	*rpl2001*	60S ribosomal protein L20a
SPAC3H5.10	*rpl3202*,	60S ribosomal protein L32
SPCC364.03	*rpl1702*	60S ribosomal protein L17
SPAC29A4.20	*elp3*	RNA polymerase II holoenzyme component (predicted)
SPAC20H4.07	*rhp57*	RecA family ATPase
SPAC17H9.19c	*cdt2*	involved in vegetative growth, involved in sporulation
SPAC11E3.05		ubiquitin-protein ligase (E3) (predicted)
SPAC4F8.01	*did4*	involved in intracellular protein transport,
SPAC22F8.04		phosphate/phosphoenolpyruvate translocator (predicted),
SPBC2F12.12c		conserved eukaryotic protein
SPAC19G12.15c	*tpp1*	trehalose-6-phosphate phosphatase
SPAC144.06	*apl5*	AP-3 adaptor complex (predicted)
SPBC6B1.06c	*ucp2*	involved in protein deubiquitination
SPAC328.03	*tps1*	alpha-trehalose-phosphate synthase [UDP-forming]
SPAPB2B4.02	*grx4*	monothiol glutaredoxin
SPBC4B4.06	*vps25*	involved in late endosome to vacuole transport
SPAC1142.01		conserved eukaryotic protein
SPAC144.03	*ade2*	adenylosuccinate synthetase
SPAC1486.01	*sod2*	manganese superoxide dismutase

Appendix 3.3: Strains that were not tested for aberrations in invasion as they were not recovered after the conversion to prototrophy. All gene descriptions are from http://old.genedb.org/genedb/pombe/, last accessed 11/07/2011

SPAC19G12.16c	*adg2*	glycoprotein (predicted)
SPAC186.02c		2-hydroxyacid dehydrogenase (predicted)
SPAC688.12c		sequence orphan, lysine-rich protein
SPAC732.02c		6-phosphofructo-2-kinase (predicted)
SPBC1703.11		conserved eukaryotic protein
SPBC106.07c	*Nat2*	N alpha-acetylation related protein
SPBC28F2.05c		xylose and arabinose reductase (predicted)
SPBC25H2.10c		tRNA acetyltransferase
SPBC25H2.15		SSU-rRNA maturation protein Tsr4 homolog 1
SPCC1183.11		MS ion channel, EF hand
SPCC11E10.06c	*elp4*	RNA polymerase II (elongator subunit) (predicted)
SPCC1393.05	*Ers1*	RNA-silencing factor
SPBC651.05c	*Dot2*	ESCRT II complex subunit, involved in vesicle transport
SPCC188.07	*Ccq1*	telomere maintenance protein
SPCC1795.10c		Sed5 Vesicle Protein Svp26
SPBC215.14c	*Vps20*	involved in vesicle-mediated transport (predicted)
SPAC17A2.09c	*csx1*	RNA-binding protein, involved in response to oxidative stress
SPBC530.06c		translation initiation factor, eIF3 (alpha subunit) (predicted)
SPBC3H7.12	*rav2*	RAVE complex subunit Rav2 , involved in cellular response to calcium ion
SPAC31A2.02	*Trm112*	protein and tRNA methyltransferase regulatory subunit
SPBPB2B2.19c		telomeric duplication, 5 predicted transmembrane helices,
SPCC584.15c		arrestin family, calcium binding protein (predicted),
SPCPB16A4.04c	*trm8*	methyltransferase (predicted)
SPCC4G3.11	*mug154*	conserved fungal protein
SPCC16C4.17	*mug123*	involved in ascospore formation
SPCC285.09c	*cgs2*	3',5'-cyclic-nucleotide phosphodiesterase, cAMP phosphodiesterase class II
SPBC660.11	*tcg1*	DNA binding protein, involved in cellular response to stress
SPAC806.05		mitochondrial ANC9 family protein
SPAC823.04	*Rrp36*	rRNA processing protein
SPAC1F12.09	*gpi17*	GPI-anchor transamidase complex (predicted)
SPCC63.02c		alpha-amylase homolog Aah3, invovled in ascospore wall assembly
SPAC13G7.12c		choline kinase (predicted), involved in phosphatidylcholine biosynthesis (predicted)
SPBC1778.01c	*zuo1*	zuotin-like protein, DNA binding (predicted)
SPAC13G6.14	*aps1*	diphosphoinositol-polyphosphate diphosphatase

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		activity, involved in inositol polyphosphate hydrolysis
SPBC31F10.15c	*atp15*	atp15, F0-ATPase epsilon subunit
SPAC13G7.07	*arb2*	argonaute binding protein 2, involved in chromatin silencing at centromere
SPBC887.10	*msc4*	mitotic catastrophe suppressor, involved in response to stress
SPBP4H10.09	*rsv1*	transcription factor (predicted)
SPBC1289.13c		alpha-1,2-galactosyltransferase
SPAC1142.07c	*vps32*	SNF7 family, involved in intracellular protein transport (predicted)
SPAC14C4.14	*atp1*	F1-ATPase alpha subunit
SPBC2A9.08c	*sec22*	involved in intracellular protein transport
SPAC644.15	*rpp101*	60S acidic ribosomal protein
SPBC3D6.04c	*mad1*	involved in spindle assembly checkpoint (predicted), involved in cytokinesis
SPCC1739.10	*mug33*	membrane anchored protein (predicted)
SPAC17A5.02c	*dbr1*	RNA lariat debranching enzyme,
SPCC576.12c	*mhf2*	involved in replication fork processing
SPBC530.05		transcriptional regulator,
SPAC20H4.11c	*rho5*	GTPase, Rho family, GTP binding
SPAC17G8.14c	*pck1*	serine/threonine protein kinase, protein kinase C-like
SPAC1805.12c	*uep1*	ubiquitin family protein
SPAC4C5.02c	*ryh1*	small GTPase, GTP binding,involved in osmotic stress response
SPAC17G6.04c	*cpp1*	cpp1, protein farnesyltransferase (beta subunit)
SPCC4G3.14	*mdj1*	DNAJ domain protein, involved in cellular response to heat
SPAC31A2.13c	*sft1*	SNARE-associated,involved in vesicle-mediated transport,
SPAC10F6.13c		aspartate aminotransferase (predicted)
SPAC6F12.06		Rho GDP dissociation inhibitor
SPBC2G5.03	*Ctu1*	cytosolic thiouridylase subunit
SPAC23C11.02c	*rps23*	40S ribosomal protein S23
SPAC23D3.11	*ayr1*	1-acyl dihydroxyacetone phosphate reductase (predicted)
SPBP8B7.06	*rpp201*	60S acidic ribosomal protein (P2A subunit)
SPAC23H3.15c		serine-rich protein, sequence orphan, SPAC25H1.01c
SPAC17G8.10c	*dma1*	ubiquitin-protein ligase (E3) (predicted)
SPAC10F6.04		involved in microtubule-based process (regulation) (predicted)
SPAC22F8.11	*plc1*	phosphoinositide phospholipase C, involved in cellular lipid biosynthesis
SPBC1718.07c	*zfs1*	involve in mating pheromone recognition pathway,

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SPAC27E2.01		alpha-amylase homolog
SPAC4G9.19		DNAJ domain protein DNAJB family
SPAPB18E9.01	*trm5*	tRNA (guanine) methyltransferase Trm5
SPAC22F3.07c	*atp20*	F0-ATPase subunit G
SPAC824.07		hydroxyacylglutathione hydrolase
SPBC947.14c		sequence orphan
SPBC32F12.08c	*duo1*	DASH complex subunit Duo1
SPBP22H7.08	*rps1002*	40S ribosomal protein S10
SPBC83.19c		sequence orphan
SPBC21C3.11	*ubx4*	UBX domain protein Ubx4
SPBC839.14c		methyltransferase
SPAC30D11.13	*hus5*	SUMO conjugating enzyme
SPBC16H5.03c	*fub2*	SUMO E1-like activator enzyme Fub2
SPBC17G9.02c		RNA polymerase II accessory factor, Cdc73 family
SPBPB8B6.05c		L-asparaginase
SPBP4H10.03	*oxa102*	mitochondrial inner membrane translocase Oxa102
SPBC146.12	*coq6*	monooxygenase Coq6
SPAC20G8.09c		N-acetyltransferase Nat10
SPCC1682.08c		RNA-binding protein Mcp2
SPCC320.04c		GTPase Gem1
SPAC4F10.07c	*atg13*	autophagy associated protein Atg13
SPBC8D2.17		alpha-1,2-galactosyltransferase
SPAC1093.06c	*dhc1*	dynein heavy chain
SPAC24B11.10c	*chr3*	chitin synthase regulatory factor Chr3
SPBC1685.11	*rlp1*	RecA family ATPase Rlp1
SPCC24B10.09	*rps1702*	40S ribosomal protein S17
SPAC17C9.07	*alg8*	glucosyltransferase Alg8
SPCC736.06		aspartate-tRNA ligase
SPAC23C4.03		haspin related kinase
SPAC18B11.07c	*rhp6*	Rad6 homolog Rhp6
SPBC29A3.18	*cyt1*	cytochrome c1
SPBC216.02	*mcp5*	cortical anchoring factor for dynein Mcp5/Num1
SPBC1861.05		carbohydrate kinase
SPBC365.10		actin-like protein Arp5
SPAC17G6.06	*rps2401*	40S ribosomal protein S24
SPBC25B2.02c	*mam1*	M-factor transporter Mam1
SPCC962.04	*rps1201*	40S ribosomal protein S12
SPAC12B10.05		metallopeptidase
SPAC23A1.06c	*cmk2*	MAPK-activated protein kinase Cmk2
SPBC776.17		rRNA processing protein Rrp7
SPBC530.11c		transcription factor

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SPBC16A3.18	*cip1*	RNA-binding protein Cip1
SPAC3C7.07c		arginine-tRNA protein transferase
SPAC23C4.06c		methyltransferase
SPBC13G1.02		mannose-1-phosphate guanyltransferase
SPAC23C4.11	*atp18*	F-0 ATPase subunit J
SPCC777.07		alpha-1,2-mannosyltransferase
SPBC21D10.11c	*nfs1*	iron-sulfur cluster assembly protein Nfs1
SPBC23E6.02		ATP-dependent DNA helicase
SPBC15D4.01c		kinesin-like protein
SPBC31F10.14c	*hip3*	HIRA interacting protein Hip3
SPBC36B7.03	*sec63*	ER protein translocation subcomplex subunit Sec63
SPCC4G3.09c	*gyp3*	GTPase activating protein Gyp3
SPAC2F7.06c	*pol4*	DNA polymerase X family
SPCC23B6.04c		sec14 cytosolic factor family
SPAC18G6.05c		translation elongation regulator Gcn1
SPAC1F7.01c	*spt6*	transcription elongation factor Spt6
SPAPYUG7.02c	*sin1*	stress activated MAP kinase interacting protein Sin1
SPBC24C6.06	*gpa1*	G-protein alpha subunit
SPBC1734.11		DNAJ domain protein Mas5
SPBP4H10.11c	*lcf2*	long-chain-fatty-acid-CoA ligase
SPAC4D7.10c		SAGA complex subunit Spt20
SPAC644.07		Rieske ISP assembly protein
SPBC14F5.03c	*kap123*	karyopherin Kap123
SPCC23B6.01c		oxysterol binding protein
SPAC7D4.04	*taf1*	Taz1 interacting factor 1
SPAC959.04c		mannosyltransferase complex subunit
SPBC3B9.11c	*ctf1*	mRNA cleavage and polyadenylation specificity factor complex subunit Ctf1
SPAC1142.05	*ctr5*	copper transporter complex subunit Ctr5
SPCC4F11.04c		mannosyltransferase complex subunit
SPCC794.12c	*mae2*	malic enzyme
SPAC5H10.04		NADPH dehydrogenase
SPAC17H9.09c	*ras1*	GTPase Ras1
SPAC4G8.10	*gos1*	SNARE Gos1
SPCC14G10.03c	*ump1*	proteasome maturation factor Ump1
SPBC887.15c		sphingosine hydroxylase
SPAPB1E7.02c	*mcl1*	DNA polymerase alpha accessory factor Mcl1
SPBC25H2.11c		bromodomain protein
SPAC23C11.13c	*hpt1*	xanthine phosphoribosyltransferase
SPAC823.16c	*mug179*	WD repeat protein Mug179
SPBC1709.01	*chs2*	chitin synthase homolog Chs2
SPBC18E5.04	*rpl1001*	60S ribosomal protein L10

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SPAC27E2.11c		sequence orphan
SPCC4B3.08		C-terminal domain kinase I (CTDK-I) gamma subunit
SPCC1753.05	*rsm1*	RNA export factor Rsm1
SPBC1A4.05	*blt1*	regulator of G2/M transition in mitotic cell cycle
SPBC18H10.02	*lcf1*	long-chain-fatty-acid-CoA ligase Lcf1
SPAC1B3.07c	*vps28*	ESCRT I complex subunit Vps28
SPBC1703.12	*ubp9*	ubiquitin C-terminal hydrolase Ubp9
SPAC3F10.17		ribosome biogenesis protein Ltv1
SPAC1952.03		cysteine protease
SPAC589.12		cell wall organization membrane protein
SPAC29E6.10c		kinetochore protein
SPBC30D10.10c	*tor1*	phosphatidylinositol kinase Tor1
SPAC607.10	*spo3*	sporulation protein Spo3
SPAC29A4.18	*prw1*	Clr6 histone deacetylase complex subunit Prw1
SPAC144.01		sequence orphan
SPAC977.05c		conserved fungal protein
SPAC10F6.12c	*mam4*	protein-S isoprenylcysteine O-methyltransferase Mam4
SPBC27B12.08		AP-1 accessory protein
SPAC8C9.07		rRNA processing protein Fyv7
SPAC1F12.07		phosphoserine aminotransferase
SPAC16A10.03c		zinc finger protein Pep5/Vps11
SPAC23A1.16c		DUF408 family protein
SPAC222.04c	*ies6*	chromatin remodeling complex subunit Ies6

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