DECONSTRUCTING WORONIN BODY FORMATION

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Summary

The Woronin body is a peroxisomally derived organelle unique to the group of filamentous fungi known as the Euascomycetes (Dhavale and Jedd, 2006). Members of this group are colonial coenocytes, with multinucleate septate tubular hyphae. Each septum features a central pore permitting unrestricted movement of cellular organelles throughout the entire syncytium. The drawback of this system becomes obvious during tip lysis: cytoplasmic pressure forces protoplasm through the pores in adjoining septa and out of the lysed tip. The Woronin body functions by blocking the septal pores of injured hyphae; its dense central core of HEX allowing it to withstand the combined turgor pressure of many adjoining compartments, and halting further loss of cellular components. HEX self-assembles to form a crystal lattice comprised of three distinct groups of residues namely Groups I, II and III. Groups II and I interact via a central salt bridge and adjoining hydrogen bonds while Group III interacts exclusively via Hydrogen bonds. Disruption of self-assembly is known to abolish Woronin body function (Yuan *et al.*, 2003).

A fundamental question in cell biology concerns how cells determine the frequency or copy number of cellular constituents. Woronin bodies are formed from peroxisomes as evidenced by a GFP marker targeted to the peroxisomal matrix. This marker reveals two distinct populations of peroxisomes; numerous small peroxisomes and less abundant large peroxisomes and the latter appear to be involved in HEX crystal nucleation and growth. The mechanism that determines these two populations is

currently unknown. Interestingly, large peroxisomes are no longer observed in the *N.crassa* HEX deletion mutant *Δhex*. This suggests that the matrix protein HEX may control peroxisome size. Furthermore, some aspect of the HEX oligomer may mediate this control.

To test this hypothesis, point mutations in each group contact were introduced into the chromosomal copy of *hex* by homologous recombination. The resultant mutants were examined by a variety of techniques designed to assess the formation of HEX oligomers, peroxisome size and Woronin body function. Surprisingly, mutation of Group I and II residues – even key salt-bridge residues - has relatively minor effects on HEX self-assembly, the formation of large peroxisomes and Woronin body function. In contrast the Group III mutant Q134E abolishes HEX self-assembly and function and large peroxisomes are no longer observed in this mutant. In addition, this Group III mutant (Q134E) has a dominant negative effect on the import of a peroxisome targeted GFP marker. Together these data support the hypothesis that the HEX-oligomer controls peroxisome size. They further suggest a model whereby Group III-dependent HEX self-assembly is initiated in the cytosol. This assembly may play a key role in determining peroxisome fate.

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List of Symbols and Abbreviations

Units of Measurement

°C	Degrees Celsius
Å	Angstrom unit, a unit of wavelength, 10–10 m, roughly the diameter of an atom; equivalent to 0.1 nm.
bp	base pairs
e	E-value, or expectation value. The number of different alignents with scores equivalent to or better than S that are expected to occur in a database search by chance. The lower the E value, the more significant the score .
g	gram
h	hour
kDa	kiloDalton. A Dalton is a unit of mass equal to 1/12 the mass of a carbon-12 atom, 1.0000 in the atomic mass scale; numerically, but not dimensionally, equal to molecular or particle weight (atomic mass units).
l	litre
mg	milligrams
M	Molar, a solution containing (w/v) the gram molar weight of the reagent i.e. Mass in grams equal to the molecular mass of the reagent.
mM	milliMoles

ml	millilitres
ng	nanogram (10^{-9} grams)
rpm	rotations per minute
s	seconds
μg	microgram (10^{-6} grams)
μl	microlitre (10^{-6} litres)
μm	micrometer (10^{-6} meters)

Reagents and Solutions

EDTA	Ethylene diamine tetra acetate
IAA	Indole Acetic Acid
SDS- PAGE	Sodium dodecacyl sulphate - Polyacrylamide gel electrophoresis
TE	Tris-EDTA buffer utilized for re-suspending nucleic acids.
VB	Vogel's medium, Basal
VT	Vogel's medium, Top
VN	Vogel's nutrient medium

Others

Δ	delta, here, indicative of a gene deletion.
et al.	*et alter* (and others)

1.0 Introduction

1.1 Background

Fungi comprise one of three Eukaryotic Kingdoms that independently evolved multicellular organization (Dhavale and Jedd, 2006). Most fungi form colonies that consist of multicellular, multinucleate, tubular filaments known as *hyphae*. The hyphal lifestyle facilitates efficient exploitation of growth substrate, its functions include: invasive growth, long distance transport and, when required, aggregation into multicellular reproductive structures (Alexopolous *et al.*, 1996).

Hyphae typically colonize substrates through a continuous process of apical extension followed by branching and fusion, or *anastomosis,* of branches resulting in an interconnected synctial network of cells known as the mycelium (Buller, 1933b, Glass *et al.*, 2004). Based on characteristic physiological and morphological traits, as well as similarity in nucleotide sequence data, fungi are grouped into four distinct evolutionary groups or phyla (Berbee and Taylor, 2001, Bruns *et al.*, 1992, Lutzoni *et al.*, 2004). Vegetative hyphae of individuals from the basal or early diverging phyla, i.e., the Zygomycetes and Chytridiomycetes, do not make septa. In contrast, the more recently evolved Dikaryomycetes, Ascomycetes and Basidiomycetes possess distinct perforate septa. It has been suggested, based on these observations, that the septate condition evolved in an ancestor common to the Dikaryomycetes (Berbee and Taylor, 2001) (Jedd, 2006). It is also hypothesized that the presence of septa may have

favoured the more recent advent of septal pore associated organelles such as the Woronin body and septal pore cap (SPC) in the Euascomycetes and certain members of the Basidiomycetes respectively (Bracker, 1967, Muller *et al.*, 1998).

In the multicellular Kingdoms Animalia and Plantae, cells communicate with their neighbours through a variety of channels including gap junctions (Sosinsky and Nicholson, 2005) and tunnelling nanotubules (Rustom *et al.*, 2004) in the former and plasmodesmata in the latter (Gallagher and Benfey, 2005). These channels are designed to allow the passage of a limited set of cellular constituents. In contrast, the septal pore of the Euascomycetes is often large enough to allow unrestricted passage of large organelles such as nuclei via mass flow of protoplasm. This interconnectedness of fungal hyphae can prove advantageous in exploiting resources such as nutrient availability over long distances: for example in the mycorrhizal Basidiomycete, *Pisolithus tinctorius* motile-tubular vacuoles are responsible for the transport of nutrients such as phosphate throughout the mycelium via the formation of transient connections through septal pores (Cole *et al.*, 1998, Sheperd *et al.*, 1993).

The fungal mycelium is divided into two regions based upon the distance from the hyphal tip at which hyphal damage fails to inhibit the growth of leading hyphae. These are: the growth zone at the periphery of the colony where hyphal compartments cooperate to support apical tip-growth at the leading edge, and the central growth zone where hyphae

grow and accumulate biomass at a comparatively slower rate and do not contribute to the colony's radial growth (Trinci, 1971).

The aseptate nature of the mycelium of early diverging fungi suggests that the septum is not a prerequisite to the successful colonization of substrate. The presence of perforate septation however, does seem to allow for a more complex level of multicellularity than is observed in members of the basal fungi. Not only does the presence of perforated septa allow for the potential cellularization of the synctium in response to ageing or injury, fungi with septal pore associated structures also form the largest and most complex reproductive structures (Alexopolous *et al.*, 1996, Dhavale and Jedd, 2006).

This thesis is concerned with investigating the organelle known as the Woronin body. Woronin bodies are highly refractive particles associated with the fungal septum. Their existence was first documented by the Russian mycologist Michael Stepanovitch Woronin in the fungus *Ascolbolus pulceherrimus* (Woronin, 1864) and subsequently named after him by the mycologist A. H. R. Buller (Buller, 1933a). Ensuing light microscopic observation by Woronin's mycologist successors led to frequent confusion in establishing the organelle's identity; it was often mistaken for other refractile particles of cytoplasmic origin (such as lipid bodies). The advent of electron microscopy resolved this dilemma; Woronin bodies are now recognized as single membrane bound electron dense cytoplasmic particles with a proteinaceous core and are known to be derived from peroxisomes (Jedd, 2006). In Euascomycetes, Woronin

bodies function as septal plugs, serving to seal injured hyphae by occluding the septal pore and initiating subsequent wall deposition (Collinge and Markham, 1985).

1.2 Function

Woronin bodies are found in Euascomycetes, including many plant and human pathogens (Markham and Collinge, 1987). The archetypal Woronin body is a peroxisome-related organelle with a dense crystalline core composed of the protein HEX (Jedd, 2006). The organelle's size and form may vary across species (viz. hexagonal in *N. crassa*, spheroidal in *Magnaporthe grisea*) however, its function remains the same: occlusion of the septal pores of injured hyphae and prevention of the ensuing outflow of cytoplasm. Experiments in *N. crassa* (inducing hyphal damage or tip lysis by means of hypotonic shock and cutting with a razor) established early on the Woronin body's main function as that of septal pore occlusion (Trinci and Collinge, 1974). These initial observations were further bolstered by quantitative results from another fungal species, *Penicillium chrysogenum* documenting the Woronin body mediated occlusion of 90% of septal pores in injured hyphae as compared to only 5% in undamaged hyphae (Collinge and Markham, 1985). Woronin body function in pore occlusion has been conserved across a diverse range of Euascomycetous fungi; electron microscope observations have revealed that following septal pore occlusion, material similar to that comprising the cell wall is deposited over the Woronin body septal pore complex. This process

results in the initiation of new hyphal tips from the occluded septum. More recently, experiments in *Aspergillus oryzae* utilizing RFP (red fluorescent protein) targeted to Woronin bodies have documented post hyphal-damage septal plugging in exquisite three-dimensional detail (Maruyama *et al.*, 2005). The *Δhex* mutant provided insights into the Woronin body loss of function phenotype; the mutant displays a total absence of Woronin bodies in hyphae and bleeds significant amounts of protoplasm following cell lysis. (Jedd and Chua, 2000, Tenney *et al.*, 2000), confirming a function in cell damage induced septal pore sealing. As a secondary consequence, the mutant is also defective in the production of asexual spores called conidia. Δ*hex* mutants in *M. grisea* (Soundararajan *et al.*, 2004) and *A. oryzae* (Maruyama *et al.*, 2005) also lack observable Woronin bodies. In addition, the *M. grisea* mutant exhibits significant defects in pathogenocity accompanied by fatality in response to Nitrogen deprivation.

1.3 Ultrastructure and Composition of the Woronin body

Woronin bodies were purified and characterized for the first time in *N. crassa* through the combined use of differential and density gradient centrifugation (Yuan *et al.*, 2003). Woronin body-enriched fractions revealed an abundant 19kDa protein, termed HEX, which on further characterization by protein sequencing was found to define a family of closely related proteins unique to the Euascomycetes (Jedd and Chua, 2000, Tenney *et al.*, 2000). In filamentous fungi, HEX oligomerizes to

form an extremely large and stable superstructure which is localized (as detected by antibody staining) to the Woronin body matrix in *N.crassa* (Jedd and Chua, 2000), *Aspergillus nidulans* (Momany *et al.*, 2002), and *M. grisea* (Soundararajan *et al.*, 2004). Aside from microscopic evidence, sequence detail has also shed considerable light on the peroxisomal localization of HEX. *hex* and its orthologs all possess consensus C-terminal peroxisome targeting signals (PTS-1), indicative of transport of the protein into the peroxisomal matrix. Expression of transgenic *hex* in *S. cerevisiae* resulted in the formation of hexagonal intra-peroxisomal protein assemblies morphologically identical to the native Woronin body core (Jedd and Chua, 2000). These experiments also provided the first evidence of HEX's capacity for self-assembly which was found to be independent of its expression in its specific host organism, an ability retained by the protein even under *in vitro* conditions (Jedd and Chua, 2000). Collectively, these results defined HEX as a key structural determinant of the Woronin body core and confirmed the peroxisomal origin of Woronin bodies.

Unlike the single version of HEX in *N. crassa*, the biosynthesis of HEX in a number of other fungi begins with a process of alternative splicing between the first and second exons producing *two* variant proteins differing by several kiloDaltons (kDa) at their N-terminus. These two variants have been reported in *M. grisea* (Soundararajan *et al.*, 2004), *A. oryzae* (Maruyama *et al.*, 2005) and *Trichoderma reesei* (Curach *et al.*,

2004, Lim *et al.*, 2001) and are thought to be the chief cause of the spheroidal Woronin body in those species.

1.4 The HEX crystal structure

The HEX crystal structure reveals the mechanism of its self-assembly. The HEX crystal lattice is divided into three groups of amino acid residues that mediate lattice assembly. These are named Group I, Group II and Group III. Group I and II interfaces are both centred on a bi-dentate salt bridge and occupy opposite faces of the HEX monomer (Figure 1.a). The Group I and II interface results in the formation of a helical filament (Figure 1.d). Such filaments are then cross-linked through the Group III interface to produce the overall six-fold symmetry characteristic of the crystal's hexagonal structure (Figure 1.e). The importance of these crystal contact residues to HEX function is illustrated by the (Group I) H39G mutant, which is unable to self-assemble the HEX crystal *in vitro* and forms aberrant spheroidal Woronin bodies (Yuan *et al.*, 2003). The abnormal HEX crystallization exhibited by these mutants results in a soluble core lacking the core density to resist intracellular turgor pressure. As a result, instead of occluding the septal pore to facilitate repair these mutant Woronin bodies bleb out of the pore along with other cellular contents. Together, these results define the mechanism of HEX assembly and demonstrate that HEX crystallinity is required for Woronin body function.

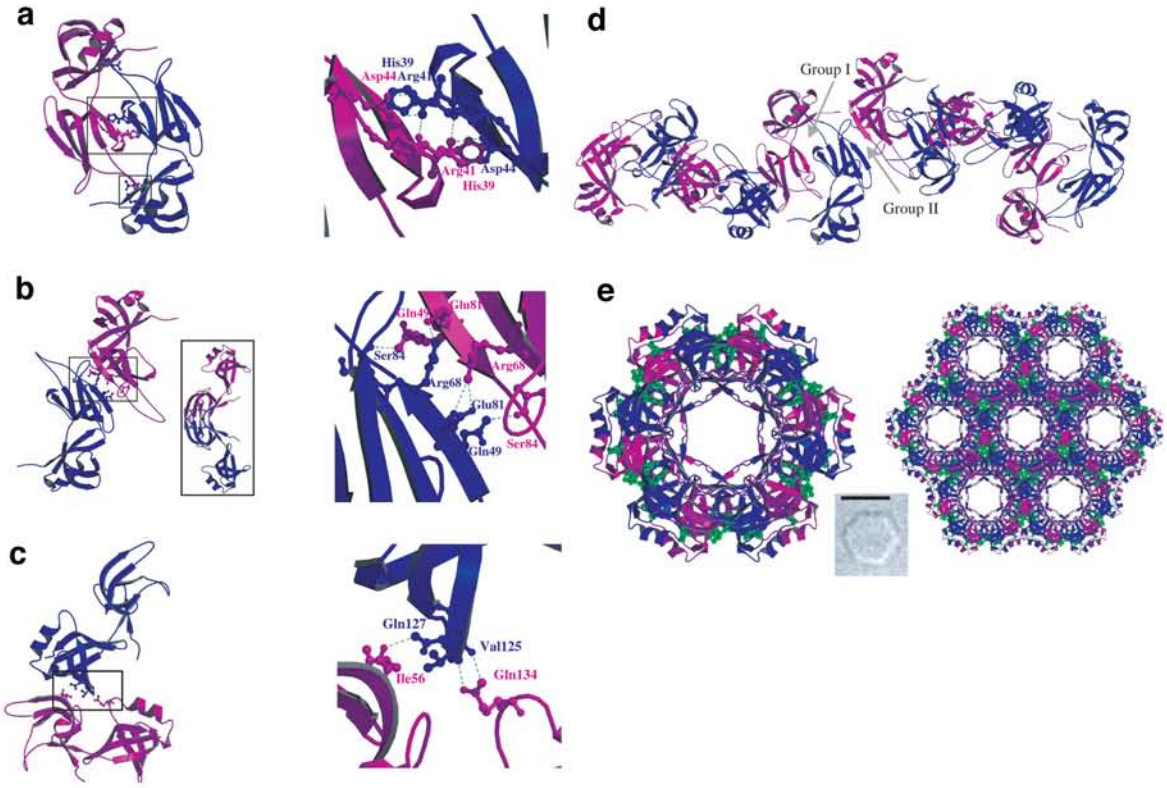

Figure 1. The HEX crystal lattice

a. Intermolecular interactions between constituent groups of HEX. Group I interaction involving central N-terminal salt bridges and the N- to C- terminal domain contacts. The His39 stabilizing the salt bridge between Arg41 and Asp44 was mutated to Gly39.
b. Group II interactions. The Glu49 hydrogen bond was mutated to Lys49 and the Arg68 salt bridge was mutated to His68
c. Group III interactions. The Gln127 and Gln134 hydrogen bonds were mutated to Glu127 and Glu134 respectively.
d. Arrangement of the HEX crystal lattice. Alternating HEX molecules in blue and purple. Coiled filment produced by Group I and II interactions. Filament depicted perpendicular to c-axis.
e. Coiled filament from (d) viewed down the crystallographic c-axis. Group III interaction residues (green) at filament surface. Inset: native HEX crystal.

1.5 Woronin body biogenesis and molecular organization of the colony

Initial hypotheses concerning an apical origin for Woronin bodies were proposed on the basis of electron microscope assisted observations of Woronin bodies being synthesized apically through progressive accumulation of electron dense material in a membranous covering (Brenner and Carroll, 1968). As Woronin bodies generally exceed septal pore diameter, it is unlikely that they are trafficked from sites of synthesis to sites of function implying these organelles are formed early in the process of hyphal growth. Initially observed by light microscopy (Buller, 1933a, Ternetz, 1900) and later by electron microscopy in a variety of Euascomycetes (Brenner and Carroll, 1968, Collinge and Markham, 1982, Momany *et al.*, 2002) Woronin bodies are consistently observed in apical compartments. In *A. nidulans* germlings that have undergone septation, fewer Woronin bodies are found at the hyphal tip, suggesting that septation may be associated with the retrograde transport and targeting of apical Woronin bodies to the septum (Momany *et al.*, 2002).

In *N. crassa,* the direct observation of Woronin body biogenesis in living hyphae via time-lapse microscopy revealed the Woronin body core in large peroxisomes moving in a tip directed manner. These mobile Woronin bodies underwent a process of maturation terminating with the organelles docking at the hyphal wall and being excluded from protoplasmic flow. This process ensures Woronin bodies are distributed roughly equally throughout the compartments of the colony (Tey *et al.*,

2005).

What factors determine the localization of this process to apical cells? Expression of a fluorescent reporter protein from *hex-1* regulatory sequences revealed a high apically localized fluorescent gradient that diminishes towards sub-apical cells. This suggested that *hex-1* gene expression is polarized to apical compartment. To determine the spatial distribution of endogenous *hex-1* transcripts, the fungal colony was fractionated into zones corresponding to apical and increasingly sub-apical hyphal compartments (Tey *et al.*, 2005). Using this system, endogenous *hex-1* transcripts were found to be enriched at the leading edge of the fungal colony, while other transcripts were shown to accumulate in the colony interior (Tey *et al.*, 2005). These experiments demonstrated the apical programming of hyphal gene expression and suggested a possible role for localized *hex-1* mRNA transcripts in the apical formation of the Woronin body. To test this model, the *hex-1* structural gene was expressed from the regulatory sequences of a transcript normally confined to the colony interior. Under these conditions, Woronin body formation was re-directed to hyphal compartments at the colony interior. Thus, the localization of *hex-1* transcripts is a key determinant of the localization of Woronin body formation. In this case, the apical programming of gene expression ensures that the first sub-apical compartment contains a complement of functional Woronin bodies (Tey *et al.*, 2005). Expression of *hex* transcripts from sub-apically expressed regulatory sequences leads to a loss of function 'bleeding' phenotype further underlining the

significance of an apical origin for Woronin bodies

1.6 Phylogenetic distribution and Evolutionary concerns

The *hex* gene is only found in Euascomycetes, which are believed to have arisen from a single common ancestor approximately 500 million years ago (Berbee and Taylor, 2001). HEX shows both structural and sequence similarity to eIF-5A (Yuan *et al.*, 2003). eIF-5a proteins are found from Archaea through Eukaryotes and are highly conserved (Krypides and Woese, 1998). Sequence comparison of *hex* to human *eIF-5a* (NCBI protein database entry gi: 9966867, blastp E-value = $3e^{-08}$), reveals 52% similarity. HEX however lacks several key features of eIF-5A such as, a highly conserved lysine, which, in eIF-5A, is modified to the amino acid hypusine vital for eIF-5A participation in the processes of mRNA metabolism (Zuk and Jacobson, 1998) and nucleocytoplasmic RNA transport (Hofmann *et al.*, 2001, Rosorius *et al.*, 1999). The structural and functional disparities between the two proteins are further highlighted by the fact that HEX fails to complement a temperature sensitive yeast eIF-5A mutant (Jedd and Chua, 2000) and that the HEX protein is targeted to the peroxisomal lumen, whereupon it displays its inherent ability to self assemble into characteristic hexagonal crystals. Conversely, despite the tendency of eIF-5A protein to form dimers, and reversible tetramers and hexamers in solution (Chung *et al.*, 1991), there is no evidence of any oligomerization (akin to that of HEX). Thus, *if* eIF-5A does self assemble, it does so through interactions distinct from those mediating HEX assembly.

Thus, HEX probably evolved following ancestral duplication of eIF-5a and acquired new functions associated with peroxisome targeting and self-assembly.

1.7 Experimental Hypothesis

The experimental hypothesis was based on the reasoning that some aspect of the Woronin body matrix is responsible for the formation of large HEX crystal forming peroxisomes. Alignment of HEX crystal contact residues with the analogous positions in EIF-5a suggests that contact residues in HEX correspond to largely conservative substitutions in EIF-5a (Figure 2.a). Based on this information individual HEX group residues were mutated to the analogous amino acid in EIF-5a consensus (Figure 2. b). The aim was to avoid disrupting the proteins overall fold while identifying the group contact(s) responsible for controlling the process of Woronin body formation.

a

Figure 2.

a. Alignment of *Neurospora crassa* (Nc_Hex) sequence with HEX sequences with the eIF-5A consensus sequence (Con eIF-5A) and the eIF-5A sequence from *N. crassa*. Roman numerals denote interaction groups I, II and III. Conserved residues highlighted in yellow. Residues identical to *N. crassa* denoted in black. Red bands indicate sallt bridges.

b

	I	II	III
SALT BRIDGES			
D44G	HR**G**SQE	QSRES	IVQQ
R68H	HRDSQE	QS**H**ES	IVQQ
D44G, R68H	HR**G**SQE	QS**H**ES	IVQQ
HYDROGEN BONDS			
Q105E	HRDS**E**E	QSRES	IVQQ
H39G	**G**RDSEE	QSRES	IVQQ
Q49K	HRDSQE	**K**SRES	IVQQ
Q127E	HRDSQE	QSRES	IV**E**Q
Q134E	HRDSQE	QSRES	IVQ**E**
Q105E Q49K	HRDS**E**E	**K**SRES	IVQQ
Q105E, Q134E	HRDS**E**E	QSRES	IVQ**E**
Q49K, Q134E	HRDSQE	**K**SRES	IVQ**E**
Q127E, Q134E	HRDSQE	QSRES	IV**EE**

b. Alignment of the mutations introduced into *N. crassa* HEX. Mutated residues are indicated in bold font. Roman numerals indicate interaction groups I, II and III. Note that mutated residues are in keeping with the Nc_eIF-5A sequence depticted in section **a**.

2.0 Materials and Methods

2.1 Neurospora crassa strains used

The strain FGSC # 9720 with the Genotype *delta mus-52:: bar+, his-3* was used for transforming mutagenized samples. Successfully transformed isolates were sequenced then crossed with the *pan-2* strain GJ344 mating type *mat a* thus creating a suitable background for transformation with the pan^{+} GFP-PTS1 construct (Tey *et al.*, 2005). For more information on strains used, please refer to *Appendix 4* for fungal strains utilized.

2.2 Stock Solutions for Media preparation

2.2.1. Trace elements solution

On a magnetic stirrer at room temperature the following reagents are dissolved in 95 ml of distilled water, with continuous stirring until such time as no precipitates remain:

Citric acid. 1 H_2O	5.0 g
$ZnSO_4$. 7 H_2O	5.0 g
Fe $(NH_4)_2(SO_4)_2$. $6H_20$	1.0 g
$CuSO_4$. 5 H_20	0.25 g
$MnSO_4$. 1 H_20	0.05 g
H_3BO_3, anhydrous	0.05 g

Na_2MoO_4. 2 H_20	0.05 g

The volume is then made up to 99 ml with distilled water, to which 1 ml Chloroform is added (as a preservative). The solution is then bottled, capped tightly and stored at room temperature.

2.2.2. Vogel's 50X salts

In 750 ml distilled water, the following reagents are dissolved in the order that they are listed in, accompanied by continuous stirring on a magnetic stirrer at room temperature.

Na_3 citrate. 5 ½ H_20	150 g
KH_2PO_4, anhydrous	250 g
NH_4NO_3, anhydrous	100 g
$MgSO_4$. $7H_2O$	10 g
$CaCl_2$. $2H_2O$	5 g
Trace Element Solution	5 ml
Biotin Solution	2.5 ml

2.2.3. Media composition (various)

(for 500ml unless otherwise stated)

	VN	VB	VT
50X Vogel's solution	10 ml	10 ml	10 ml
Trace elements	100 μl	100 μl	100 μl
Bacto-agar	7.5 g	7.5 g	7.5 g

Sucrose	7.5 g	-	7.5 g
D-Sorbitol	-	-	91 g
1X SC (without sucrose)	-	-	-
Uracil	-	-	-
Uridine	-	-	-
De-ionized water to	500 ml	450 ml	450 ml
Autoclave			
10X sugars	-	50 ml	50 ml

2.2.4. Fries' Synthetic Crossing Media (SC)

KNO_3	1.0 g
K_2HP0_4	0.7 g
KH_2PO_4	0.5 g
$MgSO_4.7H_20$	0.5 g
$CaCl_2$	0.1 g
NaCl	0.1 g
Biotin solution	0.1 ml
Trace elements solution	0.1 ml
Sucrose	1.0 g
Final pH	6.5
De-ionized water to	1000 ml

PYR-4 (supplement for the pyr-4 background of Δ*hex* mutant)

Uracil	U-0750	1g/ l

Uridine	U-3750	1g/ l

(To be added before autoclaving).

NB: in the following protocols, all media were supplemented with required nutritional supplements/selection markers unless otherwise stated. All procedures were carried out under sterile conditions (in a laminar flow hood or in close proximity to a naked flame) unless otherwise stated.

2.2.5. Microconidiation induction medium

1X Synthetic Crossing medium (without Sucrose or Agar)	50 ml
Agar	10 g
Sucrose	2.5 g
Uracil	0.5 g
Uridine	0.5 g

The volume is made up to 500 ml with distilled water and the mixture is autoclaved. Following autoclaving 5ml of filter sterilized 0.1 M Sodium Iodoacetate (Sigma) is added to the medium. The addition of iodoacetate selectively stimulates microconidiogenesis in *N. crassa* (Rossier *et al.*, 1973).

2.3 Techniques

2.3.1. Starting cultures of Neurospora crassa

5 ml slants of VN agar (with requisite supplements) are made in 50 ml falcon tubes, solidified and inoculated with either 1μl of macroconidia

(suspended in distilled water) or by contact with an inoculation wand dipped into a frozen glycerol stock of macroconidia. Incubation is carried out at room temperature for 7 days followed by harvesting conidia by suspending in 5ml distilled water, vortexing, and filtering through a 40μl mesh strainer (BD Falcon). Spores collected in this manner can then be stored at 4°C (viable for 3-4 months with decreasing germination efficiency) or used for preparing 50% glycerol stocks for storage at -80°C (indefinitely).

2.3.2. Purifying mixed cultures

Subsequent to transformation, colonies picked from densely inoculated plates can carry nuclei from wild type colonies (acquired following anastomoses with wild type colonies). Since macroconidia are often multinucleate, there are chances of macroconidia grown single colonies being heterozygous for the mutant gene. Under such conditions, one can differentiate between the mutants and the heterozygotes by diluting the macroconidial suspension 1:1000 in sterile distilled water and innoculating different concentrations suspended in VT (with requisite supplements). Spores with single nuclei exhibit clearly defined phenotypes. If this technique is unsuccessful, strains are inoculated onto microconidia induction medium to induce microconidiation.

2.3.3. Microconidiation

Under conditions of starvation *N. crassa* vegetative mycelium gives rise to predominantly uninucleate conidia known as microconidia (Baylis and DeBusk, 1967, Horowitz and Macleod, 1960). Microconidiation is a convenient (albeit, time consuming) method for obtaining pure cultures. 5ml slants of micronidiation medium (supplemented with IAA, see *Material and Methods* section for more information) are poured into 50 ml falcon tubes (with requisite supplements). Each slant is then inoculated with 1 μl of macroconidial suspension in water and kept at room temperature (with alternate light and dark exposure corresponding to a photoperiod of at least 8 hours) for a week. The microconidia are harvested by suspending in 5 ml of distilled water and vortexing vigorously for 1 minute. The suspension is then strained through a Millex SV 5 μm filter unit (Micropore) and centrifuged at 13000 rpm. The water is carefully pipetted out leaving about 2 ml behind. The microconidia are resuspended by vortexing thoroughly and then inoculated in VT at different dilutions. Pure culture single colonies can be observed under the stereomicroscope within two days of inoculation. These are then excised and inoculated on 5 ml VN slants for obtaining pure culture conidia.

2.3.4. Complementation assay

Complementation assays facilitate the identification of genes carrying specific mutations. A strain carrying a visually recognizable mutation on an unknown gene is mixed with another strain carrying a

visually recognizable mutation on a known gene. Care is taken to ensure that both strains are of the same mating type. If the mutations are on the same gene, the mutant phenotype persists in the mixture and the gene responsible for the phenotype is thus identified. A mixture exhibiting a wild type phenotype is indicative of complementation, that is, indicative of the fact that both strains have mutations on disparate genes. Complementation assays can be carried out using either conidia (suspended in distilled water) from both strains, or agar blocks of mycelium cut from VN plates containing mycelium from each strain. The latter method is preferable as it allows for the selection of equivalent amounts of mycelium of the same physiological age from both isolates.

2.3.5. Crossing

Fries' crossing medium (SC) containing the required supplements (omit all selection markers like hygromycin, BASTA, etc) and 1% agar is poured into plates at 20 ml/plate and allowed to solidify. In the case of *pan-2* mutants requiring Ca-panthothenate as a nutritional supplement a 10X concentration of pantothenic acid (10 mg/ml) is recommended (even when the protoperithecial parent is *pan+*) for improving crossing efficiency (Perkins *et al.*, 2001b). The protoperithecial (female/A) parent is inoculated onto the surface of the plate directly from the glycerol stock and allowed to colonize the entire plate surface for the next 2-3 days. During this time separate cultures of the male (a) strains are inoculated on VN agar containing requisite supplements. Agar blocks (1x1cm) are cut out

from the peripheral zone of the mating type a cultures and placed on the surface of the SC plates containing the female strain. The plates are then sealed with Micropore tape, inverted, and incubated in the dark at room temperature. Over the next 2 weeks the plates are scanned regularly for the appearance of perithecia, which, on maturing, develop beaks from which ascospores are liberated. These dark brown almond-shaped ascospores are visible on the plate cover.

In order to create a *pan-2* background for the mutants, the *his-3* strain was crossed with a *pan-2* strain. The reason being that, for clear visualization of peroxisomal dimorphism in these mutants, transformation with a GFP-PTS1 tag mobilized in the vector pOKE103 was required. However, selection for this particular GFP-PTS1 construct is pan-based, i.e., upon successful transformation with the pOKE103 containing the GFP construct, pan-2 mutants regain the ability to convert ketovaline to ketopantoic acid (Perkins *et al.*, 1973) and no longer require supplementation with Ca-pantothenate. For this reason, mutants were crossed with the *pan-2* strain FGSC # 466a. However, due to incompatibility between the two strains, all crosses were found to be non-functional, i.e., perithecia formed but failed to release ascospores. In order to by-pass this incompatibility the crossing strain was substituted with another: GJSF# 340 (also *pan-2*). The crosses were successful and the resulting ascospores were inoculated onto VB-VT supplemented with the required amount of Ca-pantothenate (for more information please refer to the section on media supplements). From the resulting single colonies,

those displaying the mutant phenotype were inoculated on VN slants (also supplemented in the same manner), their spores collected and used for innoculating cultures on VN *without* Ca-pantothenate supplementation. The spores that failed to germinate were *pan-2*. These strains were further characterized by PCR and cycle sequencing to confirm presence of the required mutation and, following positive characterization, were transformed with the vector GJP 602 containing a GFP-PTS1 tag and a *pan+* selection marker (Tey *et al.*, 2005). Positive transformants were selected based on survivability on Ca-pantothenate deficient medium, characterized by mutant phenotype and sequenced. Mutants with comparable expression levels of GFP were chosen and observed under a compound microscope (Olympus BX-51) using the corresponding fluorescent filter.

2.3.6. Harvesting ascospores

When ascospores are visible on the plate cover, the cover is removed and 3-4 ml of distilled water is pipetted into it. Under the stereomicroscope a pipette tip or an inoculating loop is used to carefully suspend all visible ascospores in the distilled water. This solution is then pipetted out into 1.5ml microtubes (Axygen). These tubes are then inoculated for an hour at 60°C in a dry bath; the inoculation serves to inactivate any macroconidia that may be present while simultaneously breaking the dormancy of the ascospores and stimulating their germination (Dodge, 1912). Varying dilutions of the spores are then

suspended in 10 ml aliquots of VT (containing requisite supplements) each of which is then poured onto pre-prepared VB plates (containing requisite supplements). In the case of Woronin body mutants, the mutant phenotype of germinating colonies is observable as early as the next day.

2.3.7. Induction of competence in Neurospora crassa conidia

A week prior to induction of competence, *N. crassa* macroconidia are inoculated on VN slants containing the required supplements. (Addition of selection markers such as hygromycin is recommended against as it adversely affects the efficiency of conidiation. For information on the conidial count method of determining efficiency of conidiation, please refer to section *2.3.16 Functional assay* in the Materials and Methods section and Appendix 3).

Slants are then incubated at room temperature for 7 days, until powdery orange conidia are clearly visible. 5 ml chilled sterile distilled water is added and the tubes are vortexed thoroughly and the suspension filtered through 40μm cell strainers (BD Falcon) into sterile Falcon tubes which are placed on ice for 30 minutes. Spores are then washed 4 times with chilled 1M sorbitol (each wash involves the following steps: Cold centrifugation at 3000 rpm for 5 minutes at 4ºC, decantation of sorbitol and addition of fresh chilled sorbitol in steadily decreasing amounts followed by vortexing to resuspend). After the first wash the suspension is transferred to a 2ml tube). Finally, the competent spores are stored at 4ºC (for a minimum of 2 hours in case of immediate transformation).

2.3.8. Transformation of Neurospora crassa

For stored spores: the spore suspension aliquots are removed from 4ºC and washed three times with sorbitol. Aliquots of 40-50ul are made and approximately 1/10th the volume of DNA (concentration 4-5ng) in TE is added. The mixture is incubated on ice for 20-30 minutes and then pipetted into an electroporation cuvette and electroporated at 2.1kV. After the electroporation pulse, 1 ml of ice-cold 1 M sorbitol is immediately added and the cuvette is incubated at 30ºC for 45 minutes. Aliquots of varying dilutions suspended in VT are inoculated on VB agar plates using the requisite selection/ supplements and incubated overnight at 30ºC.

2.3.9. Site Directed Mutagenesis of the hex gene

The *hex* gene utilized as the mutagenesis template is part of a cassette containing the entire *hex* genomic seqence downstream of a Hygromycin resistant selective marker sequence (See Appendix 1). The entire sequence, known as the *hex1.hyg.int.cassette* was cloned into the pSP72 backbone, creating the plasmid p836. Site directed mutagenesis of the *hex* gene was carried out using the STRATAGENE QuikChange®Site-Directed Mutagenesis Kit (catalogue #200518) with the oligonucleotides listed in Appendix 2. This was followed by *Dpn I* digestion (enzyme provided with the same kit, at a concentration of 10U/ μl) of p836 to digest template parental DNA thus selecting for newly synthesized mutation containing DNA. These mutagenized plasmids were then transformed into XL1Blue Supercompetent cells (available in the same kit

from Stratagene) and selected overnight at 37°C on LB agar plates containing Ampicillin (50μg/ ml). Liquid cultures (LB containing the same amount of Ampicillin) were then inoculated with single colonies, and incubated overnight at 37°C. Mutagenized plasmids were extracted using the Fast Plasmid ® Mini kit (Eppendorf). PCR was carried out using the following primers:

hex1.seq1: 5' GCCTCTACAGCGAGTCTACTG 3'

hex1.seq2: 5' CATAACATCCAAGCAGCCCT 3'

Amplification with the above primer pair results in a single band 835bp in length. This band was cut out of the gel, extracted using the illustra GFX DNA and PCR band purification kit (GE Healthcare) and suspended in T.E. Cycle sequencing was carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosytems). Sequence data was analyzed and aligned using the Seqman software (DNA*).

Positives were inoculated into liquid cultures on which plasmid prep was carried out using the Fast Plasmid® Mini kit (eppendorf). Concentrations were quantified on a Nanodrop® ND-100 spectrophotometer (quantification is done prior to restriction digestion because the presence of BSA adversely affects accuracy of readings). This was followed by linearization of the vectors by restriction digestion using Xho1 from New England Biolabs® Inc. and visualization of 1/10th of the sample on a 1% agarose gel. Samples were then combined and

ethanol precipitated to a concentration of 4-5μg in 10μl. Phenol treatment was not carried out as the presence of the restriction enzyme in the final transformation mixture is thought to facilitate integration (via restriction enzyme mediated insertion or REMI. (See Guerin and Larochelle, 2002 for a comprehensive review of the process). A solution of these linearized vectors in T.E was then used to transform competent cells of *N. crassa* strain FGSC# 9720 (*mus-52, his-3, Hyg*). The transformed cells were plated on VB-VT (supplemented with Histidine and with Hygromycin as a selectable marker) and identified based on the Woronin body deficient bleeding phenotype. Individual colonies were then grown on VN (supplemented with Histidine and hygromycin for selection and phyloxine B for visualization of the mutant phenotype) and screened by PCR for monitoring correct insertion of the construct. The primers used for screening were as follows:

P1: 5' CGTATATGCTCCGCATTGGT 3'

P2: 5' GCGAAGCACCCATAATGTCT 3'

P3: 5' GTCGACATGAAGGTCGTCCA 3'

P4: 5' CGTGGATTGGCTCTCTCATT 3'

Samples from individuals containing the insert in the correct orientation exhibited a 900bp band for the primer pair P1 and P2 and a single band at 1.9kb for the primer pair P3 and P4 respectively. Impure samples (i.e., those from colonies having undergone anastamosis with

wild type colonies), however, exhibited two distinct bands when amplified using the primer pair P3-P4: one band at 550bp and another at 1.9kb. The 550bp band is indicative of a wild type copy of the gene, lacking the hygromycin segment. Isolates exhibiting an impure genotype were purified by microconidiation while the remaining (pure) isolates were sequenced. The sequencing primers used were as previously mentioned (see previous page).

2.3.10. Cycle sequencing

The reaction mixture for cycle sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosytems) is comprised as follows:

BigDye Terminator ready reaction mix	6μl
Template (300-500ng for a sequence up to 2kb)	xμl
Primer (3.2 pmol)	1μl (10mM)
De-ionized water	to make up the volume to 20μl
Total	20μl

The above mixture is mixed well, briefly spun down and amplified using the following PCR program:

96°C for 1minute

96°C for 10 seconds

50°C for 5 seconds

60°C for 4 minutes

2.3.11. Long-term storage of cultures

Isolates with a pure genotype are inoculated on VN slants and the resulting conidia are harvested for long-term storage. 1x1cm blocks of agar with mycelium on the surface are used to inoculate 5 ml slants of VN containing the requisite supplements. Cultures are incubated at room temperature for 7 days then harvested by suspending them in sterile distilled water and pouring through a sterile 40 μm mesh strainer (BD Falcon) into a 50 ml Falcon tube. This step separates any hyphae that may be present in the suspension. The flow through is used for preparing glycerol stocks (using a 1:1 ratio of conidial suspension in water: 50% glycerol), which are then stored at -80°C. For a complete list of plasmids constructed and strains obtained in this manner, refer to Appendix 4.

2.3.12. Pre-treatment of liquid cultures of Neurospora crassa for protein extraction

10 μl of macroconidial suspension of the required *Neurospora* strain is inoculated in 5 ml of liquid VN (with requisite supplements if required) in a 250 ml conical flask at room temperature for 6 hours on a shaker at 50 rpm. At the end of this inoculation period the total volume is made up with fresh VN to 50 ml and the culture is incubated overnight on a shaker (at 50 rpm).

The following morning the mycelium is poured through a sterile 40 μm mesh strainer (BD Falcon) and transferred using fine tipped forceps between two blotting papers kept between a stack of C4 towels. After 10 seconds the mycelium is shifted to a dry area on the blotting paper and dried for 20 seconds more. It is then transferred to a 50ml Falcon tube, which is securely sealed and placed in liquid Nitrogen. (Mycelium treated in this manner can be stored for extended periods at -80°C).

2.3.13. Protein Extraction Protocol

Autoclaved mortars and pestles are cooled to room temperature and swabbed with pure ethanol then chilled using liquid Nitrogen. A spatula is chilled the same way, and then used to transfer the frozen mycelial mat to the chilled mortar. The chilled pestle is then used to grind the mycelial mat to a fine powder (continuously adding liquid Nitrogen to prevent thawing). 500 μl by volume of this powder is transferred to a 2ml micro-tube along with 1000 μl of 2X loading buffer (prepared according to standard protocols in Sambrook and Russell, 2001a). The microtube is placed in a heat block at 100°C for 5 minutes and then allowed to cool at room temperature. Samples treated in this manner can then be stored indefinitely at -20°C.

2.3.14. SDS-PAGE

SDS-PAGE was carried out according to standardized protocols from *Molecular Cloning: a Laboratory Manual* (Sambrook and Russell,

2001a) in a Criterion™ Cell System (BIO-RAD) with a 12% resolving gel and a 5% stacking gel. A pre-stained marker (10-100kD) was used for size estimation (BIO-RAD). Electrophoresis was allowed to proceed at 100 V for two hours. Two gels with equivalent loading were run simultaneously, the first was stained with Coomassie blue as per standardized protocol in *Molecular Cloning: a Laboratory Manual* (Sambrook and Russell, 2001a) the second was used for Western blotting.

2.3.15. Western Blot

The procedure was carried out as per the standardized protocol in *Molecular Cloning: a Laboratory Manual* (Sambrook and Russell, 2001b) in a Criterion™ Cell System (BIO-RAD). The transfer was carried out at 100V for 2 hours at 4°C. Detection (using HEX specific antibodies, secondary antibody tagged with HRP) was made using the Immobilon™ Western Chemiluminescent HRP substrate system (Milipore).

2.3.16. Functional assay

N. crassa strains were inoculated on 1ml VN slants containing the requisite supplements and allowed to grow to complete conidiation (7 days). Conidia were then harvested in 5 ml chilled distilled water and kept on ice until assayed. A haemocytometer (Hausser Scientific) was used to count the number of conidia per ml. Four of the smallest ruled squares in the upper left corner of the grid were used for counting conidia. The average number of conidia from three different isolates of each strain were

then used to calculate a conidial average in each mutant (See Appendix 3 for full list of calculations).

The following formula was used:

$$\frac{\text{Number of conidia in upper 4 squares}}{4} \times 25 \times 10^4 = \text{number of conidia/ ml}^3$$

$$\frac{\text{conidia/ ml}^3}{1000} = \text{number of conidia/ ml}$$

Number of conidia/ml x 5 = number of conidia/ml of VN

3.0 RESULTS

3.1 Introducing mutations into Neurospora crassa

In order to test the hypothesis that some aspect of HEX self-assembly controls the formation of large peroxisomes, mutagenesis of key HEX Group contacts was carried out. This was achieved by substitution of key residues in Groups I, II and III with corresponding residues from the eIF-5a consensus sequence (Figures 2.a and b). All mutations were introduced via site directed mutagenesis. The template used for mutagenesis was the *hex* gene in the *hex1.HYG.integration cassette* containing a genomic segment of the *hex* gene. The introduction of this construct into a strain of *N. crassa* allowed for homologous recombination of transformant DNA. The simultaneous directed integration of the *Hygromycin*^{R} gene downstream of the *hex* gene (located on chromosome 1 of the *N. crassa* genome) allowed for selection of positive transformants (Figure 3). The procedure was carried out in this manner to eliminate the possibility of mutant phenotypes caused by incorrect integration.

3.2 Steady state levels of HEX in mutants

Mutants were grown in liquid culture and extracts were prepared (as described in the section *Materials and Methods*). Western blotting was used to determine the steady-state level of HEX protein and to demonstrate that the mutations incorporated did not affect the level of HEX expression. Figure 4.b shows that all mutants (with the exception of

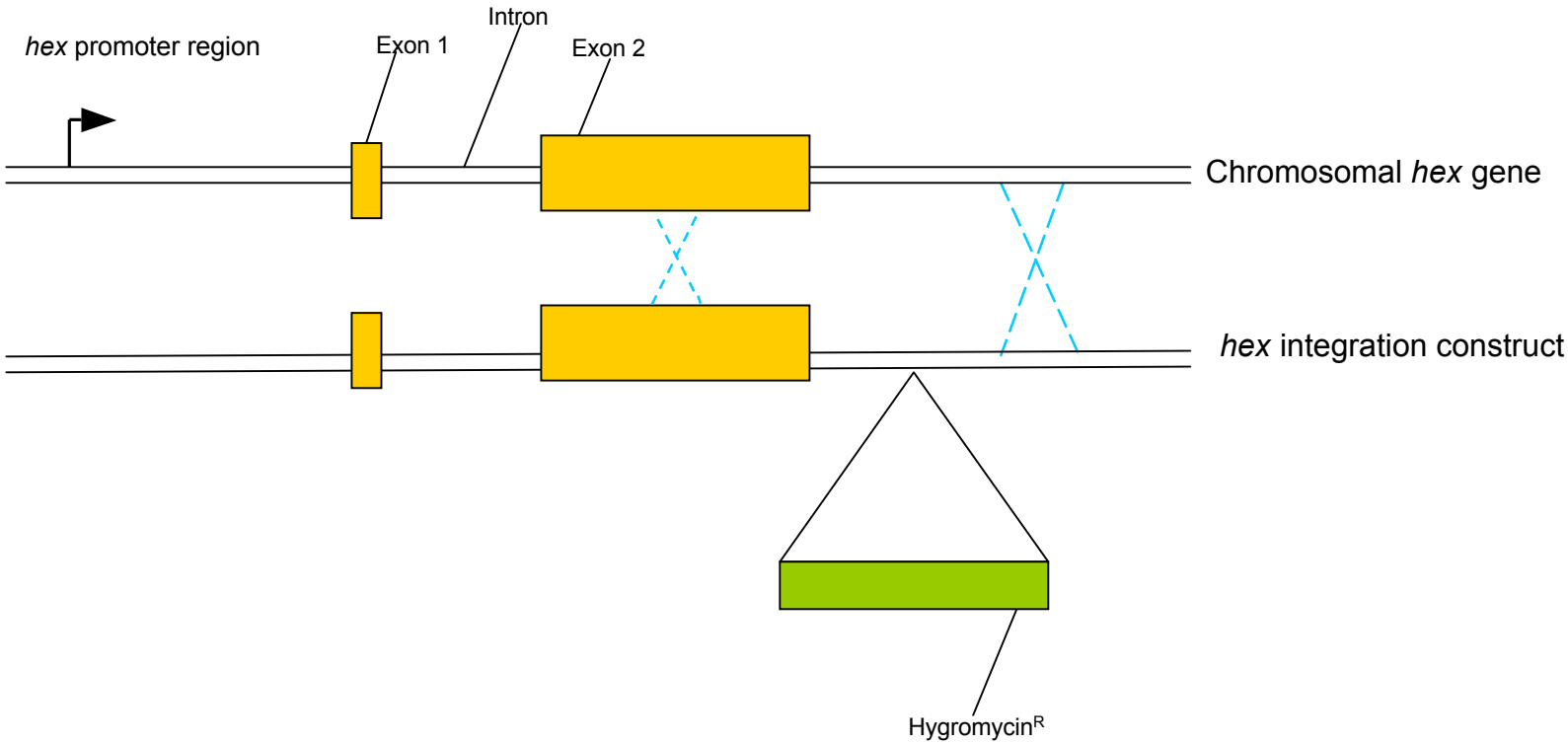

Figure 3. Construct used for targeted insertion of vector containing mutagenized *hex* gene

Δ*hex*, which was used as a negative control) displayed amounts of HEX equivalent to those present in wild type. This finding suggests that defects in self-assembly do not cause HEX turnover. Moreover, they suggest that differences in HEX crystal formation are dependent only on the physical properties of HEX.

3.3 HEX crystal morphology

3.3.1. Group I

Wild type HEX crystals assume the form of a hexagonal plate (Jedd and Chua, 2000), which can readily be observed by light microscopy (Figure 4.a). The Hydrogen bond disrupting the Group I mutation, Q105E (Figure 4.a) and the salt bridge disrupting mutation, D44G, both produce crystalline structures. Surprisingly, both of these possess a rhomboidal form (Figure 4.a).[1] This is in stark contrast to the Group I π-bond mutant H39G (this bond stabilizes the salt-bridge), which produces refractive spheroidal assemblies that are known to provide little Woronin body function (Jedd and Chua, 2000). Level of function in mutants was

[1] A series of refractive needle-like structures were also observed. However, without crystallographic data, it is unclear whether the two structures are related. Some of the needles seem to be equivalent in size to the sides of the rhomboids, while others are far too large to be part of the same structure. While the rhomboidal crystals seem to be capable of occluding septal pores, the needles themselves do not seem to possess any such property. Some of the larger needles seem, on fine adjustment, to resemble plates, although the resolution on our microscope is not high enough to document their exact nature. A matter of some interest, however, is that the needle-like structures always dock (onto hyphal walls) in the same orientation; that is, with their narrowest plane facing the observer. The structural re-arrangement responsible for this selectively oriented docking may be a result of the mutations in the Group I contacts. However, further research is required to resolve this matter.

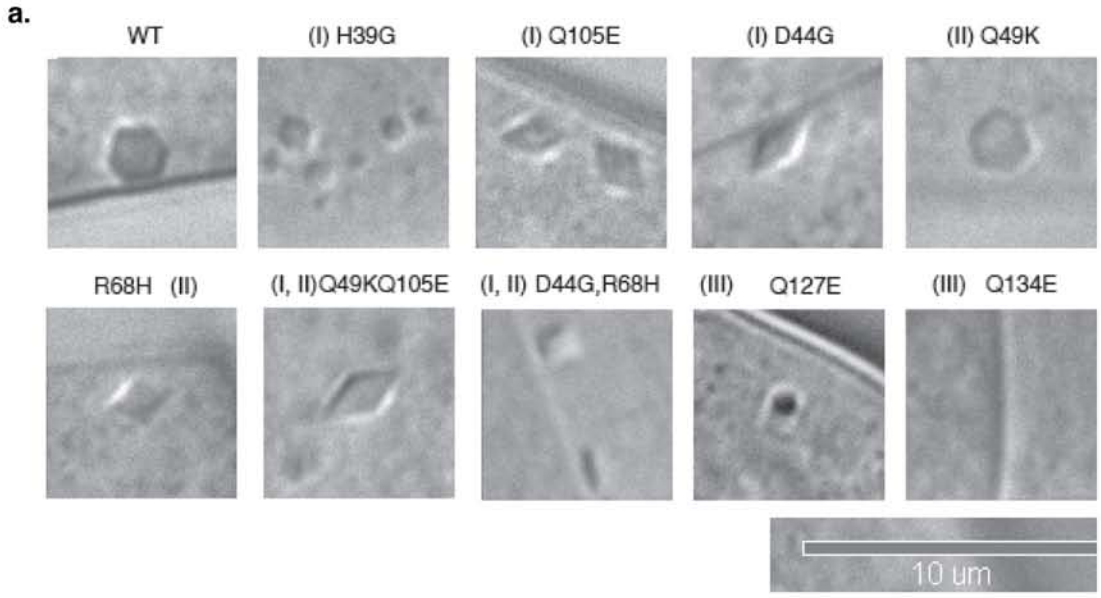

Figure 4.
a. HEX crystals observed in mutants. Roman numerals denote group number.
b. Coomassie stained SDS-PAGE gel showing equivalent loading of samples, corresponding Western blot with equivalent levels of HEX protein.

assessed by quantifying their level of conidiation and comparing it against that of the wild type (Figure 5). In HEX mutants, aerial hyphae that undergo tip lysis are unable to repair this damage and, as a result, continue to bleed protoplasm instead of making conidia (Yuan *et al.*, 2003). Q105E and D44G mutants are capable of producing relatively normal levels of conidia (Figure 5), suggesting that the rhomboidal crystalline structures retain sufficient structural integrity for function. Together, these data confirm previous work, and suggest that different mutations in the Group I interface can have very dissimilar effects on the assembly of HEX. They further suggest that alternate pathways to HEX crystallinity may be accessed in the context of certain mutations[2].

3.3.2. Group II

To further probe the structure-function relationship of HEX Group contact residues, the Group II hydrogen bond disrupting mutation Q49K and the salt-bridge disrupting mutation R68H were created. Q49K Woronin bodies are hexagonal in shape (Figure 4.a) and almost perfectly functional as is evidenced by their conidia forming ability (Figure 5). Mutation of the Group II Salt bridge contact, R68H results in the formation of a rhomboidal Woronin body similar to that exhibited by the Group I mutants, Q105E and D44G (Figure 4.a). Both Group II mutants display a

[2] Microscopic observations suggest another phenomenon particular to Group I mutants: a marked increase in the total number of Woronin bodies per hyphal compartment. Isolates of H39G, D44G and Q105E all display numerous mutant Woronin bodies docked along compartment walls as well as adjacent to septa. It is not clear at this point whether the Group I contact is essential for controlling Woronin body number. However, there does seem to be a correlation, which could be investigated further.

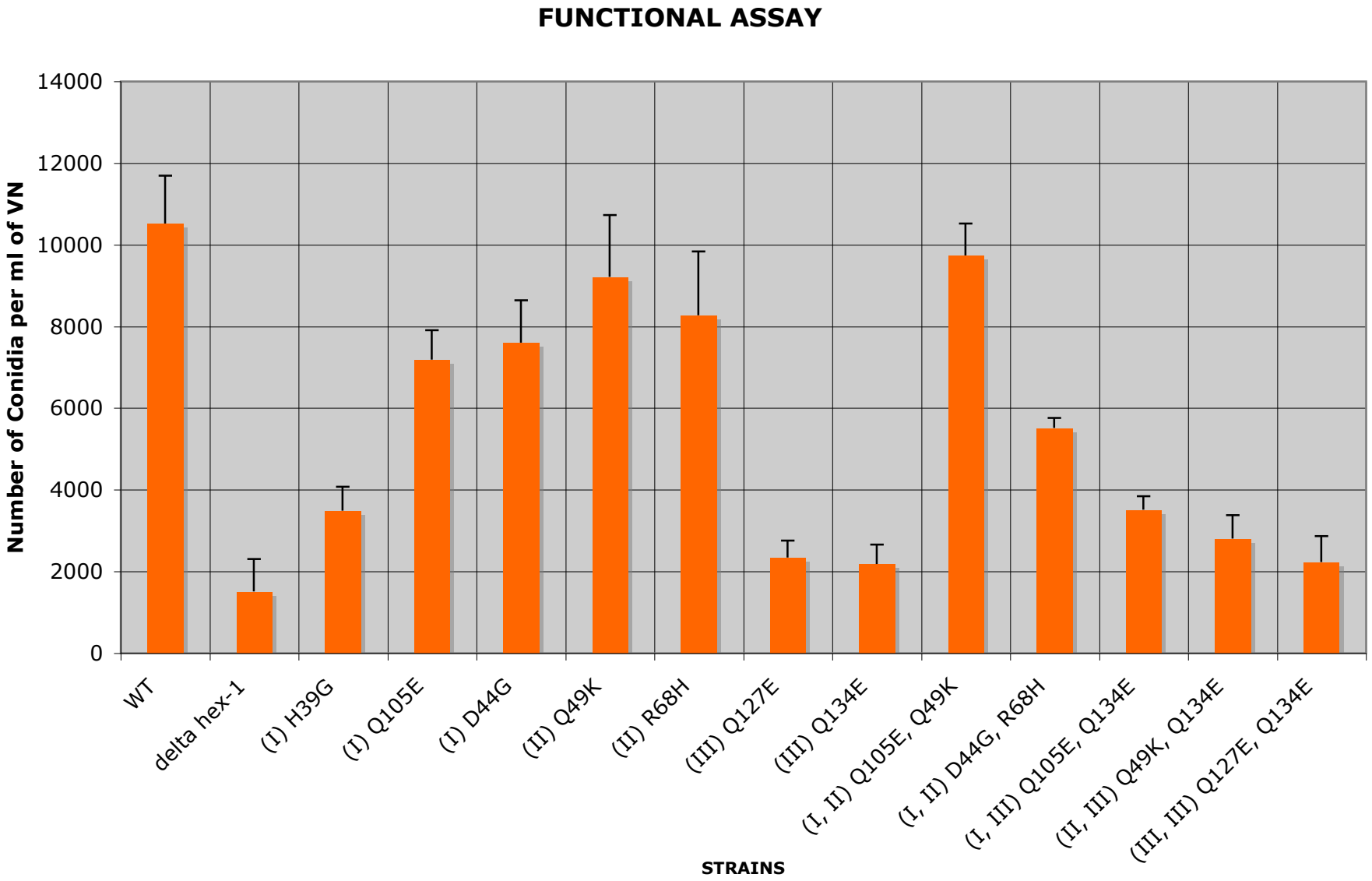

Figure 5. Graphical representation of function as depicted by conidiation frequency in various strains of *Neurospora crassa*.

slight reduction in Woronin body function, suggesting that these residues are not entirely dispensible for function. Interestingly, mutations of both Group I and Group II contacts can lead HEX to access an alternate crystal form.

3.3.3. Group III

The Group III mutants, Q127E and Q134E (Figure 4.a), exhibit the most significant effects on HEX form and function. In Q127E mutant hyphae, refractive spheroidal Woronin bodies (similar to those observed in the Group I mutant H39G) are observed. These spheroidal Woronin bodies are poorly functional (i.e. cause a measurable decrease in conidiation) and, like the spheroidal Woronin bodies seen in the H39G mutant, lack the core density to resist turgor pressure. As a result they are deformed out of septal pores following tip lysis (see Appendix 3 and Figure 5 for a numerical comparison of conidiation efficiency in different mutant strains). The Group III mutant Q134E (Figure 4.a) does not present any observable refractive structures under light field microscopy. As the mutant protein is expressed at normal levels, future experiments could utilize detection techniques such as immunofluorescence to visualize the protein's exact localization.

3.4 Double mutants

To examine the interaction between different group residues, a variety of double mutants were constructed. The combined Group I and

Group II salt bridge mutants, (D44G and R68H) while capable of producing observable refractive structures, were significantly impaired in HEX function as reflected by defects in conidiation (Figure 5). The Group III Q134E mutation produced the strongest effect: all double mutants made in combination with Q134E were phenotypically similar to Q134E, suggesting that this mutation is epistatic to the others.

3.5 Larger peroxisomes in mutants

As mentioned previously, apical cells of *N. crassa* possess a group of large peroxisomes that can grow up to 4 mm in diameter. These appear to be engaged in HEX crystal production (Tey *et al.*, 2005). To assess peroxisome size, GFP-PTS1 was transformed into Group contact mutants and examined under a fluorescence microscope. With the exception of Q134E all of the group mutants exhibit a distinct population of large peroxisomes observable in the apical compartment. In contrast, the Q134E mutant (as well as all mutants containing combinations of Q134E with any other Group contact mutant), display only one population: that of small peroxisomes. In addition, in the apical cells of Q134E mutant hyphae, GFP-PTS1 accumulates in the cytosol, suggesting that the Q134E mutant is a dominant inhibitor of GFP-PTS1 import.

4.0 Discussion

4.1 Targeting hex mutants to the endogenous hex locus

This project has developed a system for the systematic analysis of HEX mutants expressed from their endogenous chromosomal locus. All mutants were introduced to the chromosome by homologous recombination and selected through a hygromycin resistance cassette simultaneously introduced downstream of the *hex* gene (Figure 3). This is an important technical advance as it allows for the study of HEX mutant phenotypes in isolation. Western blotting confirmed equivalent protein expression levels in all the introduced mutants (Figure 4.b). As some of these Woronin body mutants have significant defects in self-assembly, the data strongly suggests against the presence of a surveillance mechanism recognizing unassembled HEX protein. Equivalent levels of protein also suggest that defects in crystal assembly are determined only by the altered character of the HEX protein and not by differences in its expression.

4.2 HEX is robustly self-assembling

Previous research has demonstrated that HEX self-assembly is a prerequisite for Woronin body function: the Group I mutant H39G (Figure 4.a) produces spheroidal Woronin bodies with a soluble non-crystalline core (Yuan *et al.*, 2003). Despite producing a refractive core, this mutant is unable to complement conidiation defects presented by the *hex* deletion

mutant, *Δhex* (Yuan *et al.*, 2003). Data from the current investigation confirms this previous analysis (Figure 5). Surprisingly, other mutants in both Group I and Group II that were predicted to have a significant impact on self-assembly did not cause as extreme an effect as did H39G. For example, the Group I and II salt-bridge residues - D44G and R68H respectively- had relatively little effect on HEX function (Figure 5). In addition, these mutants along with other Group I and II mutants produced refractive structures with a rhomboidal morphology (Figure 4.a). In the HEX crystal structure, Group I and II interfaces produce a helical filament (Figure 1.d) that is cross-linked by the Group III interaction to produce the overall hexagonal geometry of the HEX crystal. This data suggests that the Group I and II mutants are able to access an alternative mode of self-assembly and crystal packing. In this case, understanding the precise arrangement of monomers in the crystal will require resolution of mutant crystal structures.

From a biological perspective, this data demonstrates that HEX is robustly self-assembling and capable of retaining this self-assembly in a variety of mutant states. However, minor defects in conidiation displayed by these mutants as well as the more severe defect presented by the R68H/ D44G double mutant (Figure 5) suggest that these residues do play a significant role in HEX self-assembly.

4.3 The Group III mutant, Q134E is devoid of refractive structures

Group III mutants Q127E and Q134E both displayed severe effects on HEX crystal structure and function (Figures 4.a, and 5). The Q127E mutant formed spheroidal refractive structures similar in appearance to those observed in the Group I mutant H39G (Figure 4.a). Q134E also had a greater impact on HEX crystal structures to the extent that none could be observed (Figure 4.a). In addition, these mutants had the greatest impact on HEX function as supported by corresponding defects in conidiation (Figure 5). The Q134E mutant retained all these characteristics in combination with Group I, II and III mutations, suggesting that the Q134E mutation cannot be suppressed by other Group contact mutations and therefore is epistatic over all the others.

4.4 The Q134E mutant fails to form large peroxisomes and exerts a dominant effect on the import of GFP-PTS1

In order to examine the peroxisomes in the group mutants, they were transformed with a peroxisomally localized GFP marker (GFP-PTS1) expressed from the apically localized *hex* promoter. Wild type hyphae transformed with this construct displayed two distinct populations of both large and small peroxisomes in the apical compartment (Figure 6). These were also observed in all the mutants except Q134E and double mutants thereof. Moreover, a significant level of cytosolic GFP-PTS1 was observed in all mutants containing the Q134E mutation (Figure 6). In sub-apical hyphal compartments, the level of cytosolic GFP-PTS1 was found to be

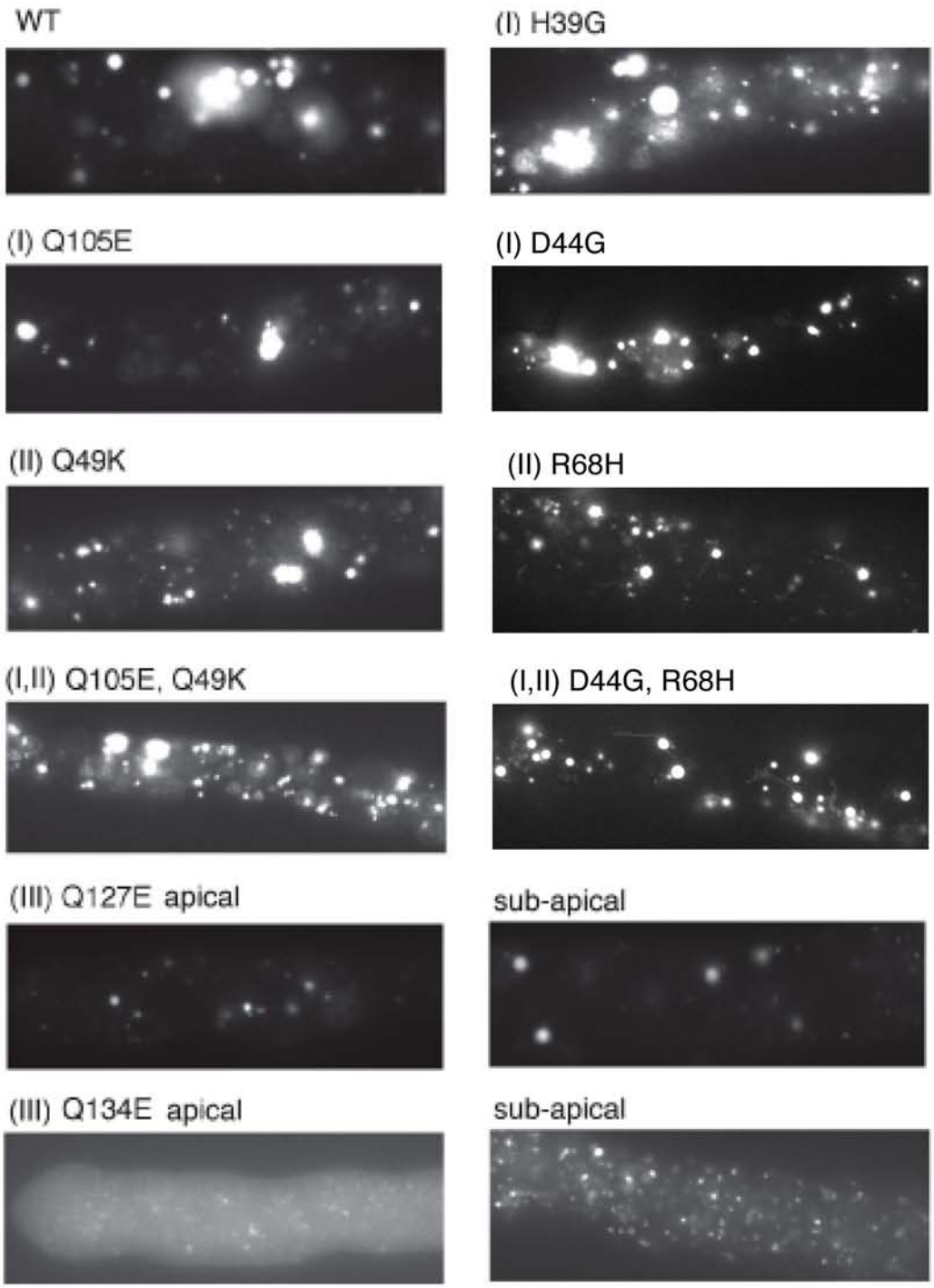

Figure 6. Localization of GFP-PTS1 to peroxisomes in Wild type and mutant hyphae. Roman numerals in brackets denote group number. Note the presence of two distinct populations of peroxisomes (small and large) visible in all strains except Q134E.

diminished and a uniform population of small peroxisomes was more clearly observable. These data suggest that the Q134E mutant has a strong dominant negative effect on the import of GFP-PTS1. Taken together, these observations imply that the Q134E mutation affects not only the cytosolic assembly and import of HEX, but also that it has a dominant effect on the import of *other* peroxisomally tagged proteins. What follows is a model explaining the process involved in these interactions (Figure 7).

In wild type hyphae, HEX monomers oligomerize in a Q134 dependent manner before being transported to the peroxisomal membrane by the carrier protein PEX5 (Figure 7). In this case, depending on the degree of HEX oligomerization, a single PEX5 chaperone may be sufficient to target many HEX proteins to the peroxisomal membrane translocation machinery. Instances of proteins 'piggy-backing' a ride on matrix-targeted proteins are well documented in various systems (Glover *et al.*, 1994, Subramani *et al.*, 2000). Since the Group III contacts are integral to HEX assembly, cytosolic oligomers are unable to assemble in their absence. Subsequently, in the absence of cytosolic oligomerization each PEX5 molecule can ferry only a single unit of HEX to the peroxisomal membrane at a time. As a result, PEX5 levels become rate-limiting for matrix localization (Figure 7). This explains the cytosolic accumulation of GFP-PTS1 in the apical compartments of Q134E expressing individuals (Figure 6). An effect on the rate of import is also confirmed by the eventual reduction of the cytosolic signal in sub-apical

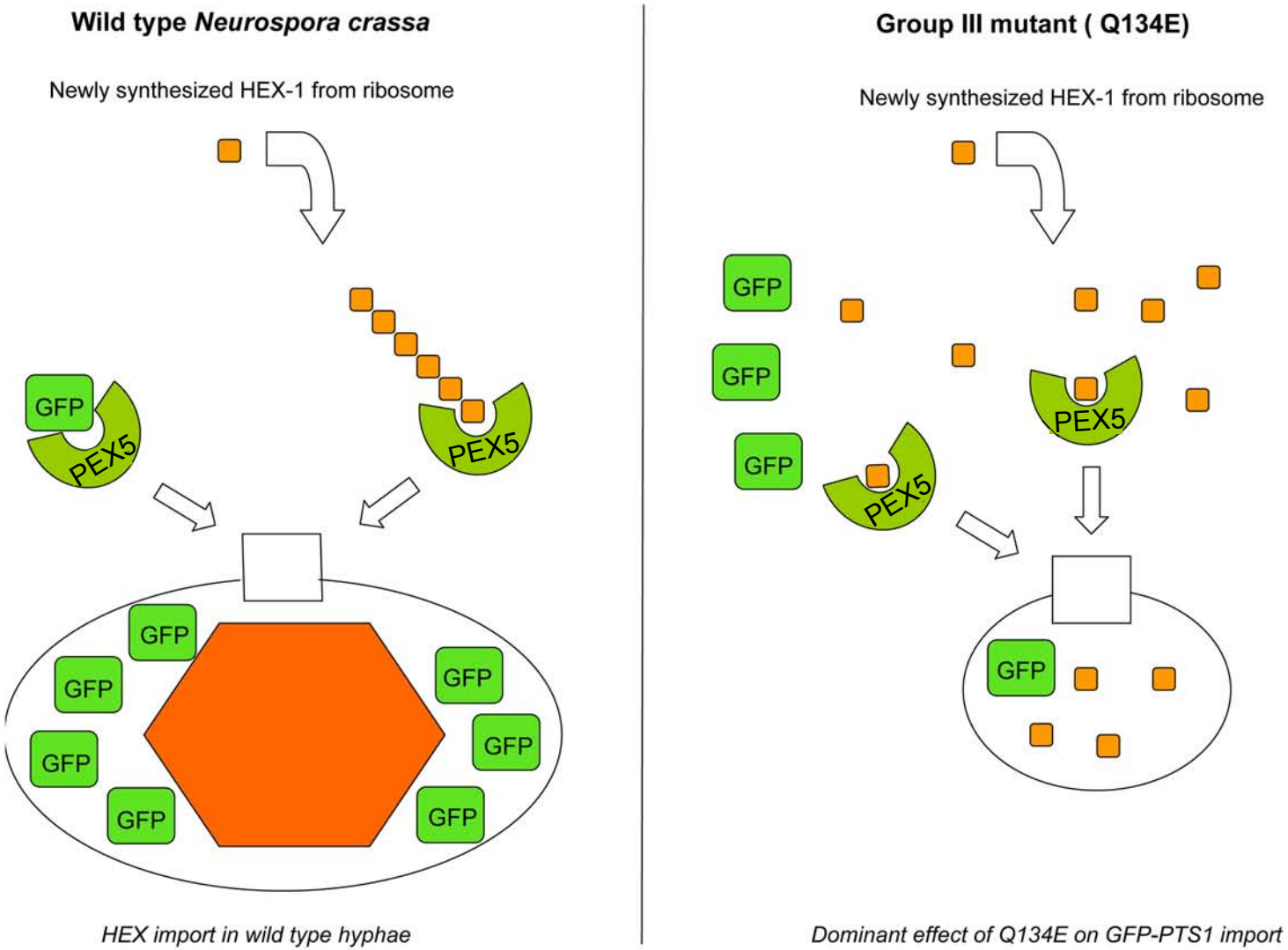

Figure 7. Model explaining the effects of the Group III mutation Q134E on protein import into the Woronin body.

hyphae, which are older and have had more time to import GFP-PTS1 into the peroxisomal matrix.

The Q134E mutant presents evidence for the importance of a Group III oligomer in the cytosol. However, the process by which the oligomer engenders increased peroxisome size is still not clear. Positive feedback between the HEX oligomer and some component of the peroxisome biogenesis machinery may provide a basis for understanding the phenomenon. In this case, the oligomer may recruit a component of the peroxisome import machinery thereby favouring the recruitment of additional HEX oligomers. Future work can be directed at addressing this hypothesis.

5.0 Conclusions

This project has investigated the role of HEX self-assembly in determining Woronin body genesis specifically in addressing the question of whether some aspect of the HEX matrix controls peroxisome size. Mutations in lattice forming residues (Group I, II and III) were systematically analyzed and the Group III residue Q134 was identified as playing a key role. While visible refractive structures and two distinct peroxisomal populations are maintained in all other mutants, the Q134E mutation eliminates both visible refractive structures and the larger population of peroxisomes. The Q134E mutation also has a dominant effect on the import of a peroxisomally targeted marker, GFP-PTS1, implying that this Group contact is important for efficient transport into the peroxisome matrix. Additionally, the Q134E mutant phenotype persists in the presence of all other group mutations indicating that Q134E mutation is epistatic over all the other mutations. These results are consistent with a model wherein the Group III oligomer promotes peroxisome growth through a positive feedback mechanism. Future work can focus on the Group III interface and cytosolic events to determine the precise mechanism(s) involved in the determination of peroxisome size.

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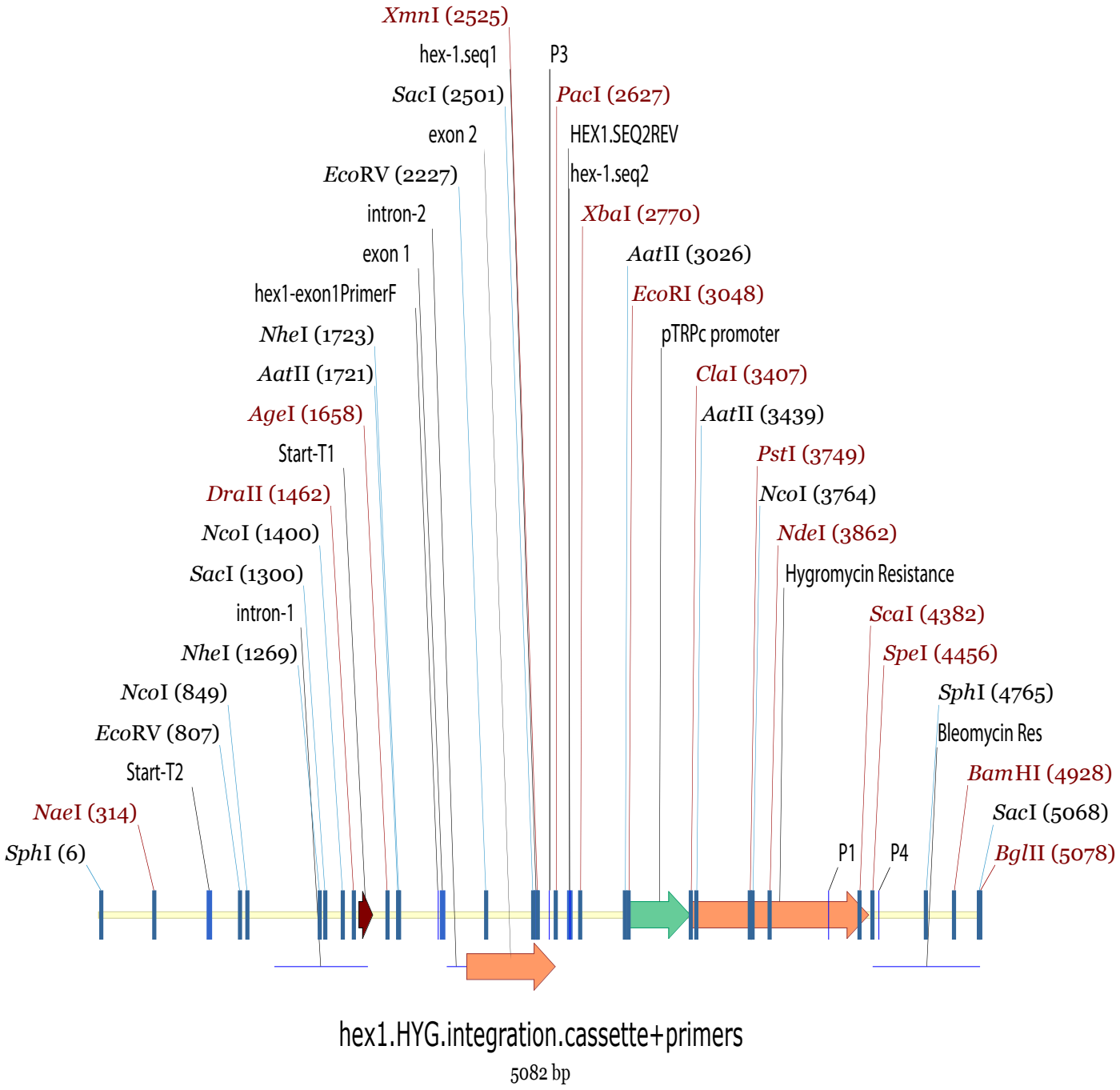

Appendix 1. Construct used for mutagenesis of group contacts in hex-1. Figure created using Vector NTI.

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                                                                  AgeI
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              AatII
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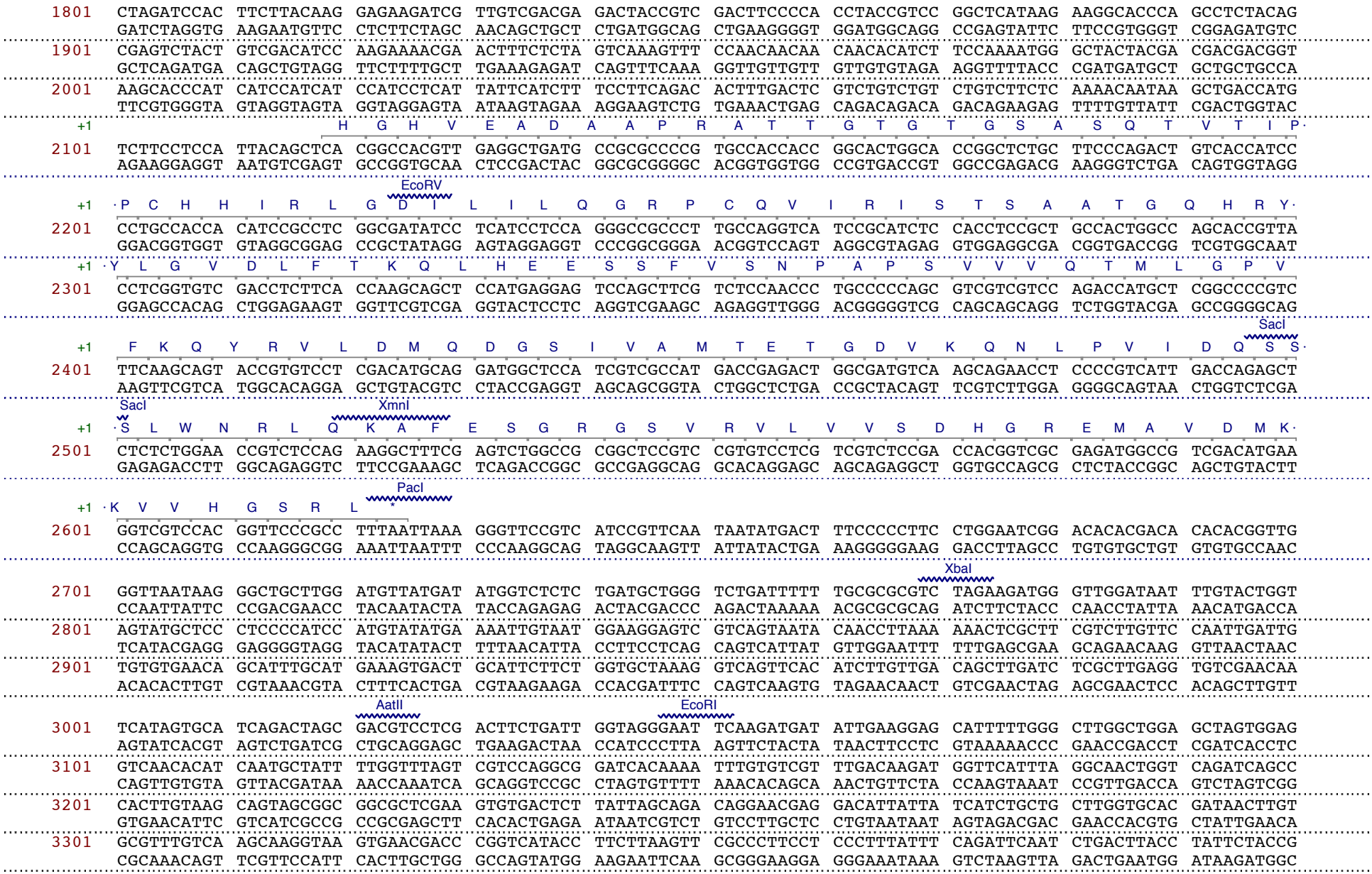
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4101  AACATCTTCT TCTGGAGGCC GTGGTTGGCT TGTATGGAGC AGCAGACGCG CTACTTCGAG CGGAGGCATC CGGAGCTTGC AGGATCGCCG CGGCTCCGGG
      TTGTAGAAGA AGACCTCCGG CACCAACCGA ACATACCTCG TCGTCTGCGC GATGAAGCTC GCCTCCGTAG GCCTCGAACG TCCTAGCGGC GCCGAGGCCC
4201  CGTATATGCT CCGCATTGGT CTTGACCAAC TCTATCAGAG CTTGGTTGAC GGCAATTTCG ATGATGCAGC TTGGGCGCAG GGTCGATGCG ACGCAATCGT
      GCATATACGA GGCGTAACCA GAACTGGTTG AGATAGTCTC GAACCAACTG CCGTTAAAGC TACTACGTCG AACCCGCGTC CCAGCTACGC TGCGTTAGCA
                                                                    ScaI
4301  CCGATCCGGA GCCGGGACTG TCGGGCGTAC ACAAATCGCC CGCAGAAGCG CGGCCGTCTG GACCGATGGC TGTGTAGAAG TACTCGCCGA TAGTGGAAAC
      GGCTAGGCCT CGGCCCTGAC AGCCCGCATG TGTTTAGCGG GCGTCTTCGC GCCGGCAGAC CTGGCTACCG ACACATCTTC ATGAGCGGCT ATCACCTTTG
                                                SpeI
4401  CGACGCCCCA GCACTCGTCC GAGGGCAAAG GAATAGAGTA GATGCCGACC GAACACTAGT ATACATAGTC AACAAGAGTC AATACACGCC AACCAATGAG
      GCTGCGGGGT CGTGAGCAGG CTCCCGTTTC CTTATCTCAT CTACGGCTGG CTTGTGATCA TATGTATCAG TTGTTCTCAG TTATGTGCGG TTGGTTACTC
4501  AGAGCCAATC CACGTCAACG AATAGGAGAG CCAAAAGTCT CGATGGTGAG TGGTAGCCGG GGATGAAGTT CAGTGGTGTC CGGTCAGCCC GTGTCTTCGG
      TCTCGGTTAG GTGCAGTTGC TTATCCTCTC GGTTTTCAGA GCTACCACTC ACCATCGGCC CCTACTTCAA GTCACCACAG GCCAGTCGGG CACAGAAGCC
4601  TCATCTCATC CTGAACCGTC TTGAAGGACA AGATACACAG GTCGAGGCAA CCACCAGGAT TGAATGAAAA TGATTCGCTC CTTCGGATAG TGGGAGAAAA
      AGTAGAGTAG GACTTGGCAG AACTTCCTGT TCTATGTGTC CAGCTCCGTT GGTGGTCCTA ACTTACTTTT ACTAAGCGAG GAAGCCTATC ACCCTCTTTT
                                                              SphI
4701  GATTGCTGTG CCGCACGGCT GCAAGCTGGA CGCGGGTCAT TGATAAGCAG CCCCTCACGG CATGCGACAC TACGAGTTCT AACCACCTTC TCAATGAGGA
      CTAACGACAC GGCGTGCCGA CGTTCGACCT GCGCCCAGTA ACTATTCGTC GGGGAGTGCC GTACGCTGTG ATGCTCAAGA TTGGTGGAAG AGTTACTCCT
4801  GAAGCCTTGA CCTCTTTCTC CAAGTACCCC CGCGTTTGAA AGGGTGAGAG TGGCAGGAGT GGTACGGGCC TCGGGCTATC AAGGGACTCA AGTCCGTGCC
      CTTCGGAACT GGAGAAAGAG GTTCATGGGG GCGCAAACTT TCCCACTCTC ACCGTCCTCA CCATGCCCGG AGCCCGATAG TTCCCTGAGT TCAGGCACGG
                      BamHI
4901  AACACCGGAT GACTGTCATA CAAATCGGAT CCCGTATCTT TTGCATGACT TTCCAGCTCC CTTTCCAGCT CCCGTCTTTG CATTATCAAC CCAAGCTGAG
      TTGTGGCCTA CTGACAGTAT GTTTAGCCTA GGGCATAGAA AACGTACTGA AAGGTCGAGG GAAAGGTCGA GGGCAGAAAC GTAATAGTTG GGTTCGACTC
                                                      SacI        BglII
5001  ATCCGACGGT GACGATTTGG AGTTTGAACT CGATCAACAC ACCAAGATAA ACCTTTTCAC TAGAGCTCAT CAGCCAAGAT CT
      TAGGCTGCCA CTGCTAAACC TCAAACTTGA GCTAGTTGTG TGGTTCTATT TGGAAAAGTG ATCTCGAGTA GTCGGTTCTA GA
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	GROUP I	GROUP II	GROUP III	DIRECTION	PRIMERS USED FOR MUTAGENESIS	PROTEIN
HYDROGEN BONDS						
	HRDSQE	QSRES	IVQQ			
Q105E	HRDS**E**E	QSRES	IVQQ	FORWARD REVERSE	GCCCCGTCTTCAAG**G****AG**TACCGTGTCCTCGAC GTCGAGGACACGGTACTCCTTGAAGACGGGGC	PVFK**E**YRVLD
H39G	**G**RDSEE	QSRES	IVQQ	FORWARD REVERSE	CATCCCCTGCCAT**GGC**ATCCGCCTCGGC GCCGAGGCGGATGCCATGGCAGGGGATG	IPCH**G**IRLG
Q49K	HRDSQE	**K**SRES	IVQQ	FORWARD REVERSE	GATATCCTCATCCTC**A****AG**GGCCGCCCTTGCC GGCAAGGGCGGCCCTTGAGGATGAGGATATC	DILIL**K**GRPC
Q127E	HRDSQE	QSRES	IV**E**Q	FORWARD REVERSE	GGCGATGTCAA**G****AG**AACCTCCCCGTC GACGGGGAGGTTCTCCTTGACATCGCC	GDVK**E**NLPV
Q134E	HRDSQE	QSRES	IVQ**E**	FORWARD REVERSE	CCCGTCATTGAC**G****AG**AGCTCTCTCTGG CCAGAGAGAGCTCTCGTCAATGACGGG	PVID**E**SSLW
Q127E,Q134E	HRDSQE	QSRES	IV**EE**	FORWARD REVERSE	CTGGCGATGTCAAG**G****AG**AACCTCCCCGTCATTGAC**G****AG**AGCTCTCTCTG CAGAGAGAGCTCTCGTCAATGACGGGGAGGTTCTCCTTGACATCGCCAG	GDVK**E**NLPVID**E**SSL
SALT BRIDGES						
D44G	HR**G**SQE	QSRES	IVQQ	FORWARD REVERSE	CACATCCGCCTCGGC**GGC**ATCCTCATCCTCCAG CTGGAGGATGAGGATGCCGCCGAGGCGGATGTG	HIRLG**G**ILILQ
R68H	HRDSQE	QS**H**ES	IVQQ	FORWARD REVERSE	GCCACTGGCCAGCAC**CAC**TACCTCGGTGTCGAC GTCGACACCGAGGTAGTGGTGCTGGCCAGTGGC	ATGQH**H**YLGVD

Appendix 2. List of primers used for mutagenesis and corresponding mutagenized proteins. Mutagenized residues are depicted in bold red font.

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FUNCTIONAL ASSAY

ISOLATES	AVERAGE	STD.DEV	Cells per ml^3	Cells per ml	Cells per ml of VN
WT	33.66666667	3.75277675	2104166.667	2104.166667	10520.83333
delta hex-1	4.833333333	2.56580072	302083.3333	302.0833333	1510.416667
(I) H39G	11.16666667	1.892969449	697916.6667	697.9166667	3489.583333
(I) Q105E	23	2.291287847	1437500	1437.5	7187.5
(I) D44G	24.33333333	3.329164059	1520833.333	1520.833333	7604.166667
(II) Q49K	29.5	4.82182538	1843750	1843.75	9218.75
(II) R68H	26.5	5	1656250	1656.25	8281.25
(III) Q127E	7.5	1.322875656	468750	468.75	2343.75
(III) Q134E	7	1.5	437500	437.5	2187.5
(I, II) Q105E, Q49K	31.16666667	2.516611478	1947916.667	1947.916667	9739.583333
(I, II) D44G, R68H	17.66666667	0.763762616	1104166.667	1104.166667	5520.833333
(I, III) Q105E, Q134E	11.25	1.040833	703125	703.125	3515.625
(II, III) Q49K, Q134E	9	1.802775638	562500	562.5	2812.5
(III, III) Q127E, Q134E	7.166666667	2.020725942	447916.6667	447.9166667	2239.583333

Appendix 3. Data used to assay function in terms of conidial number. See Figure 6. for graphical representation.

Appendix 4

Fungal strains

Strain: FGSC # 9720

Mating type: A

Genotype: *delta mus-52:: bar+, his-3*

The selection marker for this particular strain is Hygromycin added to nutrient medium at 2.5μg/μl as previously mentioned (see *Materials and Methods* for a complete description).

mus-52 is a mutation affecting the end joining of DNA. It, in effect, lowers the background of ectopic integration thus increasing the chances of achieving homologous integration. The *bar+* and *his-* genes allow for selection as follows: the *bar+* gene from *Streptomyces hygroscopicus* allows selection using the herbicide BASTA while the *his-3* blocks the phosphoribosyl AMP step in the histidine pathway, thus preventing growth on medium lacking histidine (Perkins *et al.*, 2001a).

Strain: FGSC # 466

Mating type: a

Genotype: *pan-2: pantothenic acid-2*

This strain encodes a mutated version of ketopantoate hydroxymethyltransferase that renders it unable to convert ketovaline to ketopantoic acid. *pan-2* mutants are raised on nutrient medium supplemented with 1mg/ml Ca-pantothenate. For good recovery of *pan-2* progeny, crossing media should be supplemented with 10 times the regular amount (i.e. 10 mg/ml), of Ca-pantothenate even when the protoperithecial (female) parent is pan^{+}.

Strain: GJ344

Mating type: a

Genotype: *pan-*

Individuals are unable to grow on medium lacking supplementation with Ca-pantothenate (1mg/ml). Utilized in crosses with strain FGSC#9720 (after crosses between FGSC# 9720 and FGSC#466 failed due to strain incompatibility). Its purpose was to provide the Woronin body mutants with a *pan-* background for subsequent transformation with eGFP containing a pan^{+} marker.

List of Plasmids and Mutant strains

Strains	Group No.	GJP#	GJF# (mus 52)	GJF# (pan-)	GFP-PTS1
Wt	-	N/A	71	344	48
Δhex	-	N/A	N/A	3	38
H39G	I	942	237	407	449
Q105E	I	899	242	530	539
D44G	I	1235	471	597	579
Q49K	II	900	340	541	578
R68H	II	1234	472	587	580
Q127E	III	901	147	541	555
Q134E	III	902	146	413	488
Q105E,Q49K	I, II	911	184	414	465
Q105E, Q134E	I. III	912	187	415	492
Q49K, Q134E	II, III	913	188	416	509
D44G, R68H	I, II	1276	529	598	581
Q127E, Q134E	III,III	903	253	417	480
Q105E, Q49K, Q134E	I, II, III	914	-	-	-

Appendix 5. A list of all the Plasmids and Mutant strains developed during the course of this project. GJP reflects the plasmid number in the Gregory Jedd lab plasmid list, GJF reflects the coresponding number on the fungus stock list. All plasmids were transformed into the mus 52 background before transfer into the pan- background for transformation with the pan+ GFP-PTS1 marker.