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Mitochondria and the regulation of hypervirulence in the fatal fungal outbreak on Vancouver Island

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In our recent paper, we demonstrated that the hypervirulence exhibited by a lineage of the fatal fungal pathogen *Cryptococcus gattii* is associated with its mitochondrial gene expression and an unusual mitochondrial morphology. As an important organelle, the mitochondrion has been linked to various cellular activities, but its role in modulating virulence of pathogens remains unclear. In this addendum, the potential role of mitochondria in determining virulence in eukaryotic pathogens is discussed along with future experiments that may lead to an improved understanding of this topic.

Key words: Cryptococcus, virulence, mitochondrion, intracellular parasitism, mitochondrial fusion, Vancouver Island outbreak, macrophage

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Cryptococcosis is a fatal fungal disease of humans and other animals, primarily caused by *Cryptococcus neoformans* infections in immunocompromised hosts. The related species *Cryptococcus gattii* can also cause disease, but this is generally restricted to very rare infections in tropical or subtropical areas. However, in 1999 this species was identified as the cause of an ongoing outbreak of cryptococcal disease in residents of Vancouver Island, Canada,[1] an outbreak that has since spread to mainland Canada and the northwest region of the USA.[2,3] This so-called Vancouver Island Outbreak (VIO) is remarkable for two reasons; firstly, because it represents a major expansion of *C. gattii* into a temperate area and, secondly, because most of the VIO infections have occurred in immunocompetent individuals.

Work by many groups over the last decade has clearly demonstrated that the VIO lineage of *C. gattii* is hypervirulent,[4] but the underlying molecular reasons for this hypervirulence remain unclear. However, we have recently demonstrated a potential role for mitochondrial function in regulating virulence within this organism. Our study showed that *C. gattii* strains from within the VIO lineage, but not related control strains, exhibit enhanced intracellular proliferation within host macrophages.[5] By comparing fungal gene expression whilst inside host macrophages between hypervirulent (VIO) and hypovirulent (non-VIO) strains using microarray approaches, we identified mitochondrial gene expression as the major hallmark of virulence within this species. Furthermore, we showed that VIO strains respond to the environment within the macrophage by producing long, tubular mitochondria (as opposed to the normal punctate mitochondria found in non-VIO strains during intracellular growth or in VIO strains that are grown in normal media).

In the past, the mitochondrion has been demonstrated to play a role in the fitness of microorganisms, as the organelle is essential for energy production and response to stress. For example, in *S. cerevisiae*, when mitochondria of wine yeasts were transferred to a laboratory strain, the latter showed increased viability and increased tolerance towards ethanol and high temperature.[6] Furthermore, a fungal pathogen of plants, *Heterobasidion annosum,* exhibits differential virulence depending on the mitochondrial (but not nuclear) genotype.[7] Our finding that the mitochondria of virulent *C. gattii* strains are more likely to fuse with each other during growth within host cells provides a potential explanation for the involvement

of mitochondria in virulence regulation. Since mitochondrial fusion is generally thought to protect cells from the detrimental effect of mtDNA mutations and cell death,[8-11] we proposed that the tubular morphology could be a protective response of the pathogen against the hostile intracellular environment.

Intriguingly, tubular mitochondrial formation inside host cells is very limited in less virulent strains within the same species, suggesting this trait is likely to have evolved very recently. Within the *C. gattii* species there are four genotypes, known as VGI, VGII, VGIII and VGIV, and most of the VIO strains belong to the VGII genotype. It has been demonstrated that the VGII *C. gattii* population has much lower within-lineage divergence in both the nuclear and mitochondrial genome in comparison with other groups, despite the fact that VGII is considered to be basal for the *C. gattii* species.[12] This may point to a recent bottleneck event within the VGII population, during which both mitochondrially-regulated virulence and a generally fitter population may have been selected. It is tempting to speculate that the same-sex mating event that has been proposed as the source of the VIO lineage[4] may provide just such a bottleneck during the recent evolution of this pathogen.

Although mitochondrial involvement in virulence of *C. gattii* species has not previously been proposed, indirect evidence from earlier studies suggest that the organelle might be involved in regulating virulence of another cryptococcal species, *C. neoformans*. Global in vivo transcriptional profiling of *C. neoformans* cells at the site of a central nervous system infection demonstrated that several respiratory genes were highly expressed by this yeast.[13] Two other studies have shown the importance of mitochondria in responding to hypoxic conditions and oxidative stress,[14,15] both of which occur during intracellular growth. However, a study conducted by creating stable AD hybrids to place serotype A and D mitochondria under different nuclear-genomic influences suggested that the mitochondrial genome itself is unlikely to have a significant influence on the differences between serotypes in their virulence composite in *C. neoformans*.[16] This indicates that mitochondrial virulence regulation may involve complex interactions between nuclear and mitochondrial genes.

Based on our study, the ideal experiment to test the role of mitochondrial genotype in virulence of VIO strains would be to replace mitochondria of a poor intracellular proliferator (i.e., hypovirulent) strain with those from virulent (highly proliferative) strains or vice versa. However, such an experiment is technically challenging as, unlike *S. cerevisiae*, which can produce enough ATP by glycolysis (a pathway occurring in the cytoplasm that is independent of functional mitochondria[17]) the presence of mitochondria seems to be essential to cryptococcal viability.[16] Fortunately, cryptococci exhibit a largely mating-type dependent uniparental mitochondrial inheritance: the offspring predominantly receive their mitochondria from the MATa parent, though a low level of leakage was also observed, during which biparental inheritance and mitochondrial recombination can occur.[16,18-22] This means it is theoretically possible to cross two strains (one a good proliferator and one a poor proliferator) and generate F1 progeny that contain mitochondria only from their good or poor proliferator parent (see **Fig. 1** for experimental design). In this case, the effect of mitochondrial genotype on virulence can be tested independently of nuclear genotype.

Attempts to explore this experimentally are currently ongoing in our group. Regrettably, both we and others have failed to produce viable progeny from crosses within the *C. gattii* group that contains the VIO isolates (the so-called VGII group), despite the fact the VGII strains are believed to be more fertile than the other *C. gattii* isolates.[23-25] However, crosses between groups (e.g., VGII crossed with VGIII isolates) are feasible. It has been shown that such an inter-genotype mating can result in a loss of viability in the basidiospores (<5%) and the generation of many diploid and even aneuploid progeny,[26] as the meiosis between the two genotypes is impaired because of their genomic divergence. This leads to the concern that progeny from such crosses may be generally less fit and, in addition, will contain part of the VGIII nuclear genome, which may lead to disruption of nuclear-mitochondrial crosstalk. Nonetheless, such crosses may still provide valuable information about the mitochondrial control of virulence.

Given the relatively well-conserved mitochondrial genome structure and gene synteny between cryptococci (**Fig. 2**), and the demonstration that mitochondrial genotype alone does not predict virulence in *C. neoformans*,[16] one would also suspect that the observed mitochondrial differences between virulent and avirulent strains is at least partially due to changes in nuclear-encoded proteins that affect mitochondrial morphology and gene expression. In fact, our data identified several nuclear-encoded proteins that function in mitochondria and are upregulated in the VIO strains. The nuclear-encoded mitochondrial proteins are usually synthesised in the cytoplasm and then imported into mitochondria. They interact with mitochondrially encoded proteins (e.g., in the electron transport system), control mitochondrial biogenesis, regulate mtDNA copy number, influence mtDNA stability and alter mitochondrial morphology in a sophisticated manner.[17,27-29]

Mitochondrial morphology can be affected by many genes as demonstrated by Ichishita et al.[30] In yeast and mammals, several factors including Drp1/Dnm1 and Mfn/Fzo1 are known to regulate mitochondrial morphology by controlling membrane fission or fusion.[28] Given our discovery that mitochondria in virulent (VIO) cryptococci adopt a tubular morphology during intracellular growth, such genes are prime candidates for regulators of virulence capacity in this pathogen. Interestingly, we find that *FZO1* is upregulated in the VIO strains. Fzo (*Fuzzy onions* gene), first isolated from a screen for genes involved in Drosophila spermatogenesis, is the first molecule to be identified in regulating mitochondrial fusion.[31] It is known as mitofusin and Fzo1p in mammals and yeast respectively.[32,33] The protein contains a GTPase domain (exposed to the cytoplasm) at the N-terminus and a bipartite transmembrane domain (which spans the mitochondrial outer membrane twice) near the C-terminus.[34,35] In *S. cerevisiae*, the mitochondrion of *fzo1*Δ mutants is highly fragmented due to ongoing fisson[32,36] and overexpression of Fzo1p alters the fusion/fission protein ratio and thus inhibits cell

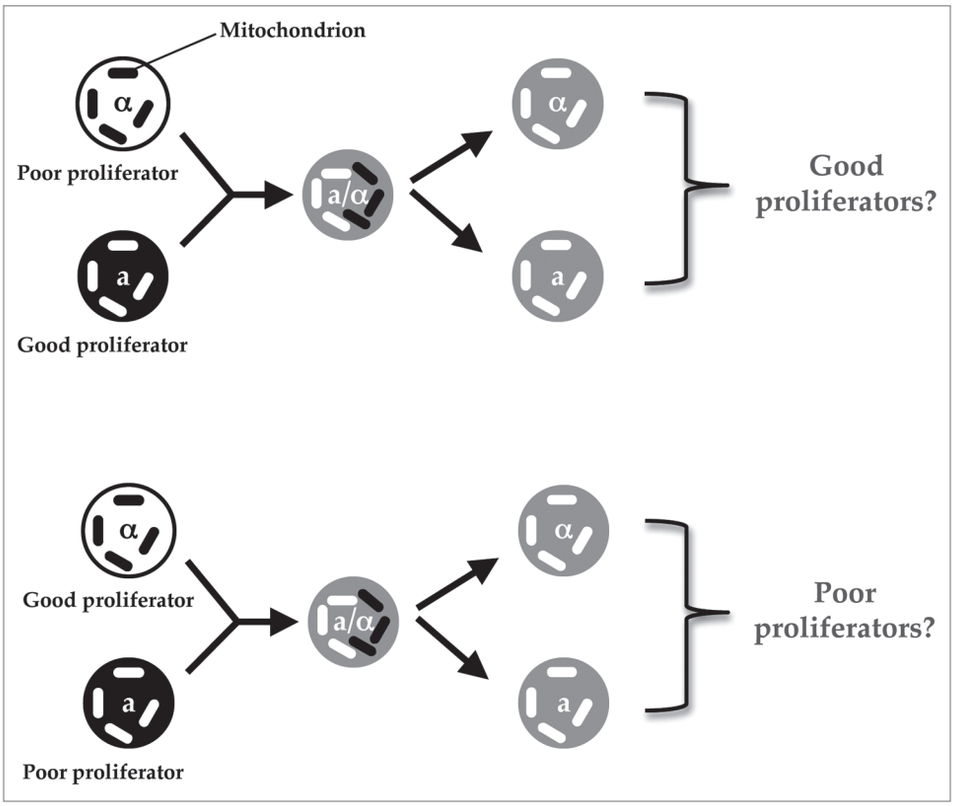

Figure 1. A schematic illustration of the experimental design for two crosses to generate progeny with mitochondria from only their mating type a (MATa) parent.

apoptosis.[11] Therefore, higher amounts of Fzo1p in VIO strains could be responsible for the tubular formation of mitochondria and also lead to a higher fusion/fission protein ratio, which is essential to increase the resistance of mitochondria and cells to apoptotic stimulation. The key unanswered questions are how nuclear-encoded proteins regulate mitochondrial activities and what external factors induce such regulation. To answer these questions, we are now subjecting the VIO strains to simple environmental stimuli commonly present in phagosomes in order to test whether these are sufficient to trigger mitochondrial morphology changes in vitro. In parallel, we are knocking out genes involved in mitochondrial fusion and fission (such as *FZO1*, *MMM1*, *MDM10* and *MDM12*,[28]) to examine whether these proteins are the regulators of nuclear-mitochondrion communication and thus hypervirulence.

To summarise, our study reveals an interesting link between the mitochondrion and virulence of *C. gattii*. Since mitochondria are found in all eukaryotic pathogens, and have been implicated in virulence in at least one other fungus (*H. annosum*),[7] mitochondrial regulation of pathogenicity may be a widespread phenomenon. More detailed experiments are therefore urgently needed to provide a clearer understanding of how mitochondria fulfil such a role.

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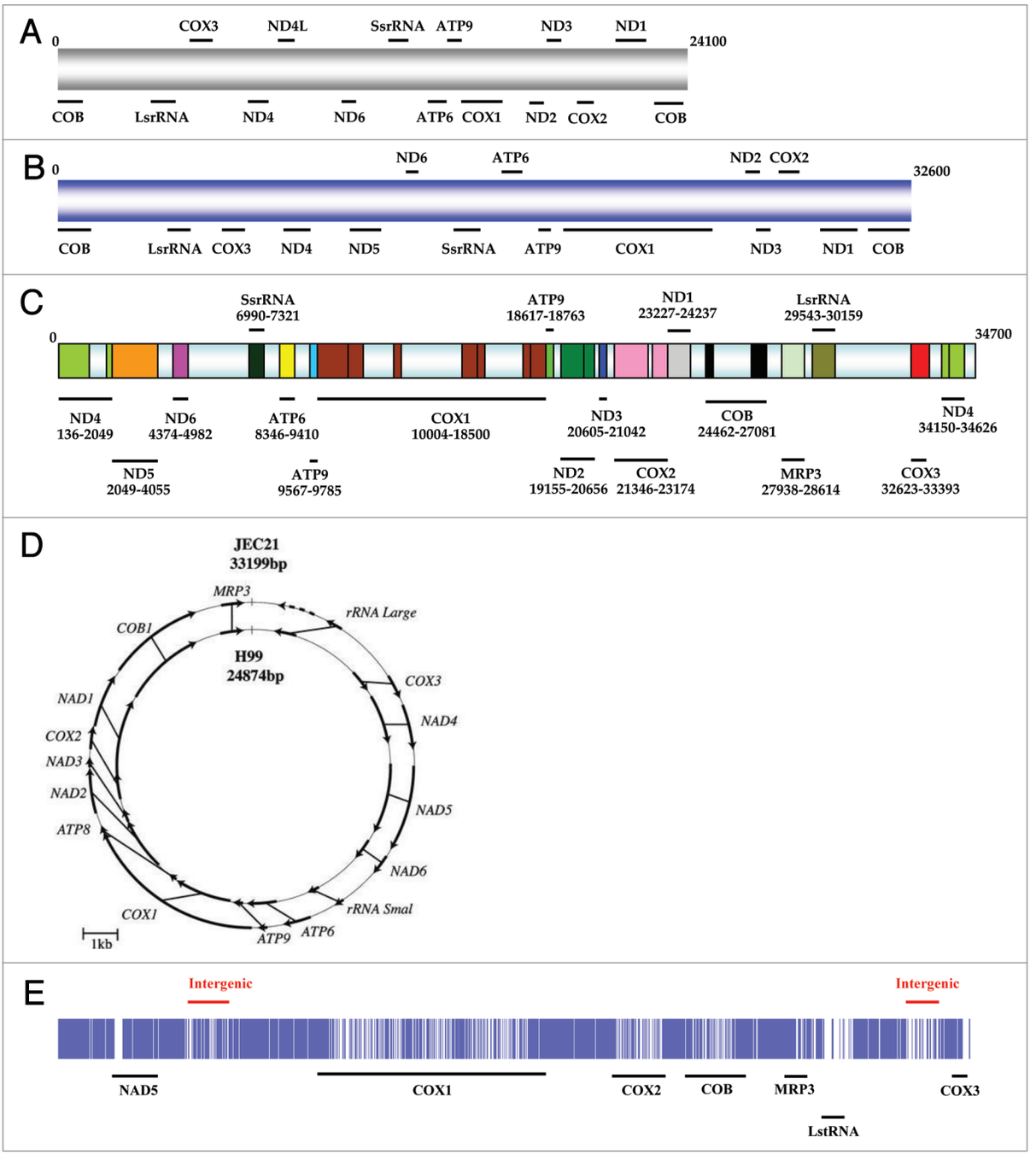

Figure 2. mtDNA structure of Cryptococcus: all the mtDNAs show a conserved gene synteny but have different sizes. (A) IFM5844 (*C. neoformans* var. *neoformans*, serotype D) and (B) IFO410 (*C. neoformans* var. *grubii*, serotype A). These two mtDNA structures were drawn to scale based on information from Litter et al.;[37] (C) mtDNA structure of A1M-R265 (*C. gattii*, VGII). Sections with light blue colour are either introns or intergenic spaces;[5] (D) Circular mtDNA structure of JEC21 (*C. neoformans* var. *neoformans*) and H99 (*C. neoformans* var. *grubii*) (taken from Toffaletti et al.[16]); (E) Simple alignment of A1M-R265 and WM276 (*C. gattii*, VGI) mtDNA using ClusterW. Before alignment, two repeat regions in both mtDNAs were removed (region one: 2434 nucleotides in COX1 gene; region two: 963 nucleotide at the end of the supercontig). Sections with white color stand for the variations between two mtDNA sequences.

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