Opinion

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Fungal evo–devo: organelles and multicellular complexity

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Peroxisome-derived Woronin bodies of the Ascomycota phyla, and the endoplasmic reticulum (ER)-derived septal pore cap (SPC) of the Basidiomycota, are both fungal organelles that prevent cytoplasmic bleeding when multicellular hyphal filaments are wounded. Analysis of Woronin body constituent proteins suggests that these organelles evolved in part through gene duplication and co-opting of non-essential genes for new functions, indicating that new organelles can arise through typical evolutionary mechanisms. Interestingly, clades possessing the Woronin body and SPC also produce the largest and most complex multicellular fungal reproductive structures. Certain Woronin body and SPC mutants have defects in growth and development, suggesting functions beyond cellular wound healing. I argue that studying these specialized systems will help to reveal the basis for fungal diversity and provide general principles for co-evolution of organelles and multicellular complexity.

Cellular adaptation and fungal evolution

Eukaryotic cells are distinguished from their prokaryotic ancestors by a complex ensemble of functionally interconnected membranous organelles, including the nuclear envelope, endoplasmic reticulum, Golgi apparatus, endosomes, lysosomes and peroxisomes. Unlike mitochondria and plastids, these organelles are not believed to have an endosymbiotic origin. Functional studies in model systems from yeast to animals suggest that the core machineries of organelle function are highly conserved, implying that rudimentary forms of these organelles were present in the ancestral eukaryote prior to the evolution of plants, animals and fungi [1,2].

Cellular organelles continued to evolve as eukaryotic lineages adopted distinct lifestyles. The fungi have radiated to produce at least seven major phyla [3] and, although their life histories are diverse, most grow through tip extension of cellular filaments called hyphae [4]. As hyphae grow, they branch and fuse [5] to produce a syncytial cellular network. Vegetative hyphae in early diverging fungi, such as Mucoromycota and Chytridiomycota, do not frequently produce septal cell walls. However, septum deposition is a primary characteristic of later-diverging Ascomycota and Basidiomycota, suggesting that this trait evolved in a common ancestor of these phyla (Figure 1a) [6,7]. Septation in hyphae is similar to cytokinesis in yeast, but with a key difference – the hyphal septum retains a central pore that links the cytoplasm of adjacent compartments (Figure 1b) and allows cooperation and coordination of activities. The pore allows fungal saprotrophs and pathogens to invasively colonize 3D substrates, permits long-distance nutrient transport [8], and supports extremely rapid hyphal tip-growth, which in some species can exceed 1 μm per second.

In the course of evolution, septal pores of both Ascomycetes and Basidiomycetes came to harbor distinct membrane-bound organelles. The best-characterized of these are the peroxisome-derived Woronin body [9] of the Pezizomycotina, the clade of filamentous Ascomycota, and the ER-related septal pore cap (SPC) of the Agaricomycotina in the Basidiomycota [10] (Figure 1c and d, respectively; for more information on the phylogenetic history of fungal organelles see [11]). The restricted phylogenetic distribution, unique structure and lifestyle supporting function of pore-associated organelles suggest that they are good models for investigating the evolution of organelles and multicellular complexity.

Initial characterization of Woronin bodies

Although a filamentous cellular syncytium supports the fungal lifestyle, it also embodies risk, which pore-associated organelles function to ameliorate. Early experiments showed that Woronin bodies occlude the septal pore in response to cellular damage [12]. Electron microscopy (EM) further showed that vesicles accumulate at the plugged septum and new cell wall material is deposited over the pore-occluding Woronin body [12], suggesting a complex pathway consisting of organelle activation, pore occlusion, plasma membrane re-sealing and initiation of polarized growth (Figure 1g).

When viewed by thin-section electron microscopy (TEM), Woronin bodies present an electron-dense proteinaceous core that is circumscribed by a closely appressed unit membrane (Figure 1c, inset). Overall size varies from 100 nm to over 1 μm and this dimension always exceeds the diameter of the septal pore [9]. In most species Woronin bodies are found on both sides of the septum (Figure 1c) and appear attached to the pore through a mesh-like tether [13]. The presence of a tether was further suggested by experiments with laser tweezers which showed that Woronin bodies pulled away from the septum recoil to their original position upon release [14].

Function and atomic structure of the Woronin body dense core

Biochemical purification of Woronin bodies allowed identification of the structural protein, HEX, which self-assembles to produce the organelle's dense core. Purified

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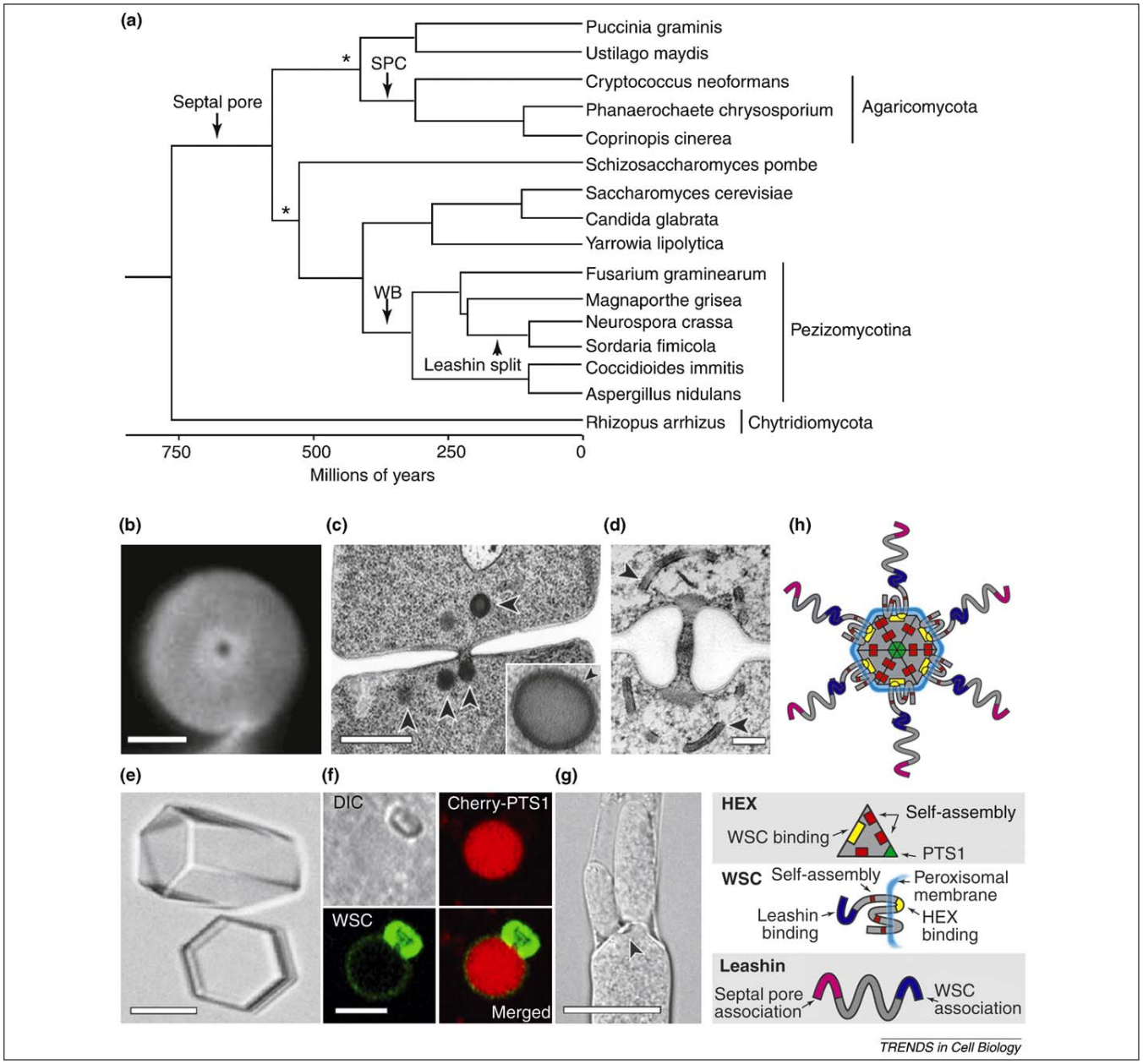


Figure 1. **(a)** Phylogenetic tree of the fungi and selected cellular traits. The likely origins of the perforate septum, septal pore cap (SPC), Woronin body and splitting of the Leashin tether are indicated. The Agaricomycota, Pezizomycota and Chytridiomycota are labeled. Asterisks mark the origin of the Ascomycota and Basidiomycota. Tree reprinted with permission from [6]. **(b)** An isolated septum from *Neurospora crassa* showing the septal pore. Scale bar, 2 μm. **(c)** TEM of Woronin bodies (arrowheads) tethered at the septal pore in *Aspergillus nidulans*. Scale bar, 250 nm. The inset shows a mature Woronin body with the membrane visible (arrowhead). Images reprinted with permission from [13]. **(d)** TEM of septal pore cap (arrowheads) in *Schizophyllum commune*. Note the electron-dense organelle core. Scale bar, 100 nm. Image courtesy of Wally H. Müller. **(e)** The Woronin body structural protein HEX self-assembles to form hexagonal rods *in vitro*. Scale bar, 10 μm. Image reprinted with permission from [15]. **(f)** Woronin bodies bud from the peroxisome matrix. A HEX assembly [seen by differential interference contrast (DIC) imaging] buds out of the peroxisome matrix (cherry–PTS1: PTS1 fused to cherry fluorescent protein) with help from the WSC membrane protein. Scale bar, 2 μm. Image reprinted with permission from [23]. **(g)** A Woronin body sealed septum from which new growth has been initiated. Two hyphal tips are growing from the Woronin body (arrowhead) sealed septum. Scale bar, 10 μm. Image reprinted with permission from [15]. **(h)** Cartoon depicting the Woronin body comprising HEX, WSC, Leashin and membrane derived from the peroxisome. Different colors are used to denote properties of these proteins, which are identified in the lower panels. Red symbolizes self-assembly, the interaction between HEX and WSC is yellow and the interaction between WSC and Leashin is shown in blue.

recombinant HEX spontaneously assembles into a 3D protein lattice (Figure 1e). *hex* mutants are devoid of visible Woronin bodies and bleed protoplasm from septal pores following cellular wounding, directly confirming an adaptive function at the septal pore [15–18].

The HEX crystal structure reveals three evolutionarily conserved surface regions governing intermolecular contact, termed groups I, II and III. A group I mutation that disrupts HEX self-assembly, when expressed as the sole version of HEX *in vivo*, results in aberrant Woronin bodies possessing a soluble non-crystalline core [19]. These organelles can attain normal dimensions but are nonfunctional [19]. The requirement for a solid core can be understood in the context of intracellular turgor pressure associated with hyphal growth [20,21]. This turgor pressure needs to be resisted during pore sealing, and this need for resistance

could have favored the evolution of HEX self-assembly and dense core formation.

Woronin body biogenesis by peroxisomal protein sorting

Three genetic loci are known to be required specifically for Woronin body assembly. These encode the core matrix protein HEX, its membrane receptor WSC (Woronin sorting complex), and the cytoplasmic tether Leashin (Figure 1h). Woronin body biogenesis takes place in the growing apical hyphal compartment through a process determined in part by apically biased *hex* gene expression [22]. Newly synthesized HEX is imported into the peroxisome matrix via its peroxisome-targeting signal 1 (PTS1) where it self-assembles to produce micrometer-scale protein aggregates. These aggregates are enveloped by the four-pass peroxisomal transmembrane protein WSC, resulting in production of asymmetric budding intermediates (Figure 1f) [23]. The relationship between HEX and WSC coordinates Woronin body morphogenesis and inheritance [23]. Specifically, HEX recruits WSC to nascent Woronin bodies and WSC in turn promotes cell cortex association in a manner dependent upon its expression level in the membrane. WSC mediates organelle segregation by recruiting the cytoplasmic protein Leashin which binds to an unidentified determinant at the cell cortex [24]. In this way, cortex-associated Woronin bodies are immobilized and retained in sub-apical compartments. The continuous execution of this process ensures that each hyphal compartment is endowed with these organelles. In the absence of WSC or Leashin, Woronin body maturation is blocked and peroxisome-trapped HEX assemblies accumulate in apical hyphal compartments [23,24]. Leashin proteins of the Pezizomycotina are over 5000 amino acids in length and contain poorly conserved repetitive sequences. These sequences are believed to determine the spacing between the Woronin body and septal pore, which is between 100 and 200 nm (Figure 1c), and to confer elasticity on the tether [24]. After cell cortex association, membrane fission is mediated by the conserved peroxisomal membrane protein, PEX11 (peroxisomal biogenesis factor 11), separating newly formed Woronin bodies from their mother peroxisomes [25]. These studies indicate that Woronin bodies are complex organelles that form within peroxisomes but mature to become physically and functionally distinct organelles. The analysis of Woronin body constituents is beginning to shed light on their genetic origins and their function in hyphal multicellular organization.

Genetic origins and evolution of the Woronin body

Several key genetic events are likely to have contributed to the evolution of the Woronin body biogenesis pathway.

Gene duplication

Remarkably, the 3D fold of HEX is nearly identical to that of eukaryotic translation initiation factor 5A (EIF5A) proteins [19]. These ancient cytoplasmic proteins are present from Archaebacteria through to eukaryotes and play a role in polypeptide chain elongation during translation [26,27]. HEX also has significant sequence similarity to EIF5A [15], suggesting that these two gene families are related by ancestral gene duplication (Figure 2a) [19]. Comparison of these proteins leads to a plausible model of Woronin body evolution. Based on their ancient origins, both peroxisomes and EIF5A were present in the ancestral filamentous Ascomycete (Figures 2a and 3a). Following *eIF-5A*

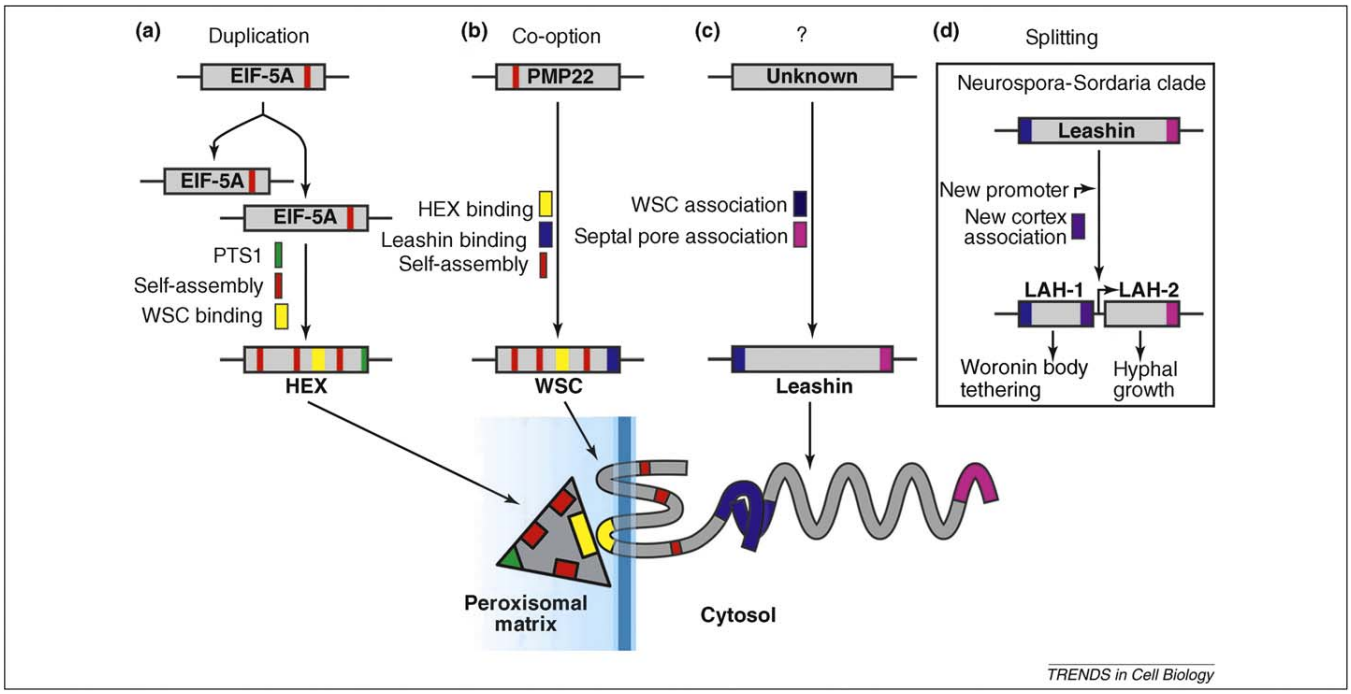


Figure 2. Speculation on genetic events associated with Woronin body evolution. **(a)** *hex* evolution via *eIF-5A* gene duplication **(b)** *wsc* evolution by *pmp22* co-option. **(c)** *leashin* evolution from an unknown precursor. **(d)** Splitting at the *leashin* locus produces two genes *leashin-1* (*lah-1*) and *leashin-2* (*lah-2*) and allowed a change in organelle localization in the Neurospora–Sordaria clade. Colors are used to denote mutations associated with the indicated protein functions. A cartoon depicting the complex formed by these proteins is shown at the bottom.

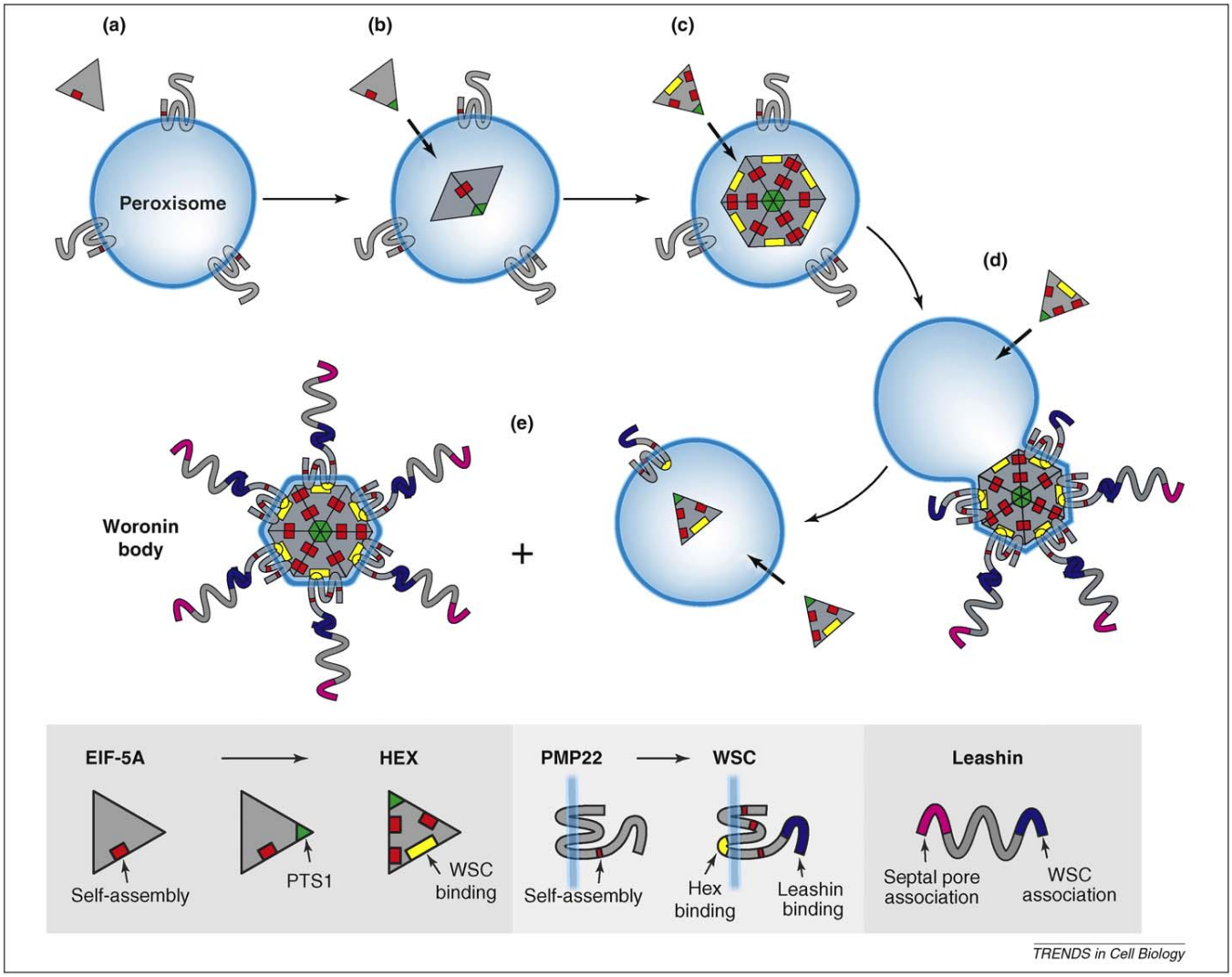


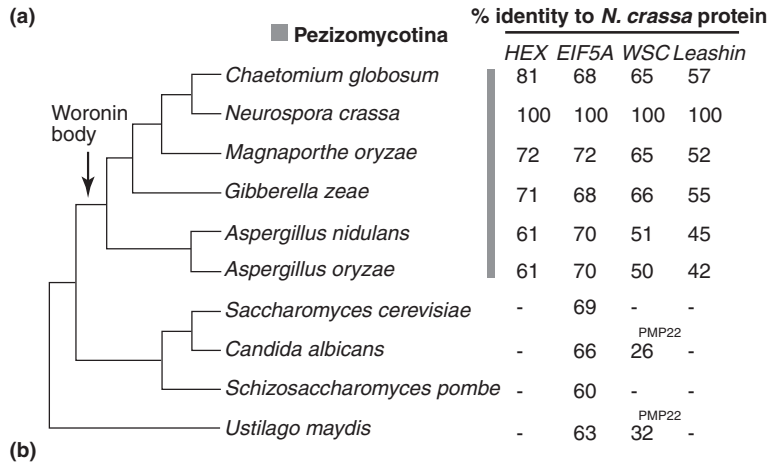
Figure 3. Model depicting cell biological changes associated with evolutionary emergence of Woronin body dedicated functions. **(a)** The ancestral state: Based on their deep phylogenetic distribution, peroxisomes, PMP22 and EIF5A were present in the ancestral filamentous Ascomycete. **(b)** *eIF-5A* undergoes gene duplication and one copy acquires PTS-1 to produce primitive HEX. **(c)** HEX self-assembly evolves through mutations affecting the three surface regions that govern intermolecular contacts (the three red domains indicated in HEX). **(d)** PMP22 co-opted to WSC function by acquiring the ability to interact with HEX oligomers and form higher order oligomers (three red domains indicated in WSC). Leashin evolves the capacity to bind WSC and the cell cortex through novel N- and C-terminal domains, respectively. **(e)** Fission of Woronin bodies from their mother peroxisomes exploits the pre-existing peroxisome division function PEX11 (for simplicity not shown). Proteins shown are identified in the legend at the bottom. Note that, for simplicity, HEX, WSC and Leashin functions are depicted sequentially, however, these were likely to have co-evolved and the order of their advent is unknown.

gene duplication, one copy acquired a PTS1 targeting signal (C-terminal tripeptide, typically SKL). Based on the sequence of contemporary EIF5A proteins, this could have been achieved through a single amino-acid substitution, resulting in primitive HEX (Figures 2a and 3b).

At least 12 specific amino acid substitutions were required to evolve HEX self-assembly from EIF5A [19]. This raises the question of how primitive HEX could persist and become fixed in a population if so many independently acquired mutations are required for its function. The answer probably lies in initial partial functionality and incremental improvements in assembly over time. Interestingly, EIF5A forms a dimer that can be modeled on the group I HEX oligomer [28] and has been reported to reversibly form tetramers and hexamers [29]. Thus, although more complex scenarios can be envisaged, weak self-assembly of primitive HEX could have immediately provided adaptive function, allowing for fixation and increasing functionality through subsequent mutations (Figures 2a and 3c).

Co-option

The HEX receptor WSC – identified from a forward genetic screen for Woronin body loss-of-function – encodes a 4-pass transmembrane protein in the PMP22 (peroxisome membrane protein 22)/MPV17 (myeloproliferative leukemia virus 17) gene family [23]. Close homologs are found in all sequenced Pezizomycotina – presumably orthologs (Figure 4a). Elsewhere, Neurospora WSC is most closely related to the PMP22 peroxisomal membrane proteins of animals, plants and fungi. The relationship between Neurospora WSC and fungal PMP22s is incongruent with known evolutionary relationships [23]. For example, Neurospora WSC is more closely related to PMP22 from



(b)

Woronin body interactome

Protein	Function	Ref
HEX	Dense-core assembly / WSC recruitment	15, 23
WSC	Budding and inheritance	23
Leashin	Tether for inheritance	24
Soft / Pro40	Hyphal fusion and sexual development	36, 37
Tmpl	Redox requlation	38
ApsB	Microtuble dynamics	39

Figure 4. Woronin bodies evolved before radiation of the Pezizomycotina and the Woronin body interactome. **(a)** The phylogenetic tree is constructed from selected fungal species with sequenced genomes. Using the indicated Neurospora proteins, BLAST searches identify homologs of Woronin-body-associated proteins in all of the Pezizomycotina, but not outside this group (percent sequence identity is indicated). WSC identifies PMP22 in *Candida albicans* and *Ustillago maydis* but not in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* from which this function has apparently been lost. **(b)** The Woronin body interactome. The table lists Woronin-body-associated proteins along with a brief description of their function. Proteins directly involved in Woronin body biogenesis are shaded in grey.

the Basidiomycete *Ustillago maydis* than to PMP22 from the Ascomycete *Candida albicans* (Figure 4a) [23], suggesting that these proteins are not evolving under strict functional constraints. In addition, PMP22 coding sequences are absent from the genomes of some fungi, including *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Figure 4a), suggesting a non-essential cellular function. These observations indicate that PMP22 might have been present in the ancestor of the Pezizomycotina where it was available for co-option into the new WSC function (Figures 2b and 3c). Initial experimentation suggests functional divergence of WSC and PMP22. PMP22 from Ustillago is targeted efficiently to the Neurospora peroxisome but is unable to envelop HEX assemblies or complement a WSC deletion mutant (Liu, F. and G.J., unpublished observation). Therefore, WSC appears to possess new and divergent biochemical properties, including self-assembly, HEX oligomer binding [23] and engagement of Leashin [24] (Figures 2 and 3). Interestingly, electrophysiological measurements have recently shown that PMP22 can form channels – believed to consist of PMP22 oligomers – for the transport of small molecules across the peroxisome membrane [30]. This property could have predisposed PMP22 to evolve the ability to form even larger oligomers required for WSC functions in Woronin body morphogenesis and inheritance [23].

Gene splitting

The Leashin tether provides a third dedicated component of the Woronin body biogenesis machinery. Variation in this tether can account for variation in organelle positioning. Leashin is conserved within the Pezizomycotina (Figure 4a) but does not have significant sequence similarity to known proteins, precluding speculation on the initial acquisition of this function (Figure 2c). Interestingly, the pattern of Woronin body cell cortex association varies systematically. In most of the Pezizomycotina, Woronin bodies are tethered directly to the septal pore (Figure 1c). By contrast, a derived, delocalized pattern of cortex association has recently evolved in a group defined by Neurospora and Sordaria (Figure 1a). In Neurospora, the *leashin* locus encodes two related proteins (Figure 2d). The 5′ gene, *leashin-1,* is required for Woronin body inheritance and encodes a protein that localizes to the Woronin body through a physical interaction with WSC [24]. By contrast, the 3′ gene, *leashin-2,* is not involved in inheritance, and encodes a protein that localizes to the septal pore and to the Spitzenkörper [31], a vesicle supply center at the hyphal apex. Remarkably, production of a chromosomally encoded Leashin-1/Leashin-2 fusion protein is sufficient to create the ancestral pore-tethered pattern in Neurospora [24,32]. This suggests that changes in Leashin are responsible for the evolutionary shift in organelle localization. These changes include evolution of promoter sequences for expression of Leashin-2 and a new cortex-association domain at the C terminus of Leashin-1 (Figure 2d).

What prompted the shift in Woronin body localization? In nature, Neurospora appears to be the first fungus to colonize burnt vegetation soon after forest fires [33]. This

ecology could explain evolution of unusually rapid growth, which can exceed 1 μm per second [34]. Both Neurospora and Sordaria are characterized by rapid growth and associated protoplasmic trafficking through septal pores. These activities could be incompatible with Woronin body pore tethering and could have favored the delocalized pattern of cortex association [35].

Evidence for additional Woronin-body-associated functions

Leashin-2 mutants are defective in hyphal growth [24] and Leashin-2 is localized to both the Spitzenkörper and septal pores. These findings suggest that the tether has an additional function in coordinating the activity of apical and sub-apical hyphal compartments. Other proteins have been localized to the Woronin body (Figure 4b), including the Sordaria Pro40 (protoperithecia 40) protein [36] and its Neurospora homolog Soft [37], which are required for fruiting body formation and hyphal fusion, TmpL (transmembrane protein L), a transmembrane protein involved in redox-related signal transduction [38] and a regulator of microtubule dynamics, ApsB (anucleate primary sterigmata B) [39]. Although the significance of their association with the Woronin body remains to be established, these findings imply that Woronin bodies have evolved to participate in diverse fungus-specific processes.

The Septal pore cap

Biochemical purification of the SPC from the filamentous Basidiomycete *Schizophyllum commune* recently identified SPC constituents [40–42]. One of these, SPC33, possesses sequences associated with ER localization and a deletion strain does not develop obvious SPCs, indicating that SPC33 is required for SPC differentiation from the ER. Interestingly, following cellular wounding the deletion strain bleeds more protoplasm than wild-type strains [42]. Thus, Woronin bodies and the SPC provide functionally convergent but evolutionarily independent solutions to the problem of septal pore gating. SPC33 mutants also have defects in colony growth and in the development of reproductive structures, suggesting additional functions in multicellular development [42]. By TEM, septal pores of the Agaricomycota sometimes contain electron-dense pore-occluding material [10]. Another SPC constituent, SPC18, can be localized to the SPC and to this cytoplasmic aggregate, suggesting that these plugs are derived from the SPC [41].

Mechanisms of organelle evolution

Based on current knowledge of septal-pore-associated organelles, some general themes begin to emerge.

The evolution of pore-associated organelles is conservative

Woronin bodies originate in the peroxisome and the basidiomycetous SPC differentiates from the ER, suggesting that when selective pressures arise for the evolution of new organelle functions, pre-existing organelles are exploited as sources of membrane and translocation machineries. Further evidence of a conservative process is seen in the use of the peroxisome division protein PEX11 [43] for the fission of nascent Woronin bodies [25]. In addition, both HEX and WSC make extensive use of self-assembly and their hypothesized precursors form limited oligomers, suggesting a predisposition for the evolution of self-assembly. SPC markers, which have only recently become available [41,42], will make it possible to determine the mechanism of SPC formation and contrast SPC and Woronin body biogenesis and function.

Typical evolutionary mechanisms can account for new organelle function

Phylogenetic and structural analysis of proteins associated with vesicular membrane deformation and membrane fusion suggests that multiple rounds of gene duplication and functional divergence provided for primary expansion of the eukaryotic endomembrane system [1,2,44]. At least three new functions were required for emergence of the Woronin body biogenesis pathway, and the evolution of these functions appears to have required substantial degrees of mutation. The origins of HEX, WSC, and the splitting of Leashin can be explained through gene duplication, co-option of pre-existing genes to new function, promoter evolution and mutation (Figure 2). The primary origin of Leashin remains enigmatic. However, if Leashin function is evolving rapidly its mechanism of origin might have been obscured by substantial mutation.

Analysis of core eukaryotic organelles, and the more recently evolved Woronin body, indicates that their evolution depended on the advent of new and inter-related protein functions, much like those of HEX, WSC and Leashin (Figures 2 and 3). In addition, it appears that substantial mutation can produce remarkable transformations in protein function, even when the overall protein fold is highly conserved, as with HEX and EIF5A. It follows that the evolution of organellar systems required substantial degrees of mutation at multiple loci. Because these emerging functions are not likely to have been genetically linked, co-evolution promoted by meiotic recombination and independent assortment of rare beneficial mutations is likely to have played a central role in the emergence of new organelle function.

Organelles and multicellular complexity

Ancestors of multicellular eukaryotes [1,45] and the Pezizomycotina (Figure 4) appear to have possessed functional organelles before they underwent evolutionary radiation. This suggests that organellar systems could constitute key innovations [46] and determinants of evolutionary success. In terms of species number and ecological diversity, the Woronin-body-producing Pezizomycotina comprise a highly successful clade. This monophyletic group is estimated to comprise 90% of the Ascomycetes and 50% of all fungal species [47]. Phylogenomic profiling suggests that a history of genetic innovation underlies this success [48,49]. Examination of approximately 5000 gene families across 33 fungal genomes identified over 25% of these genes as Pezizomycotina-specific [48]. Many of these, such as *hex*, *wsc* and *leashin*, are found in all the Pezizomycotina, suggesting that they were present at the onset of radiation

[48]. In the future, these Pezizomycotina-specific genes are likely to yield additional Woronin-body-associated functions, as well as other adaptations.

Fungal groups with the most complex and prominent septal pore-associated organelles also produce the largest and most complex multicellular reproductive structures, such as truffles (*Tuber borchii*) of the Pezizomycotina, and the widely cultivated mushrooms (*Agaricus bisporus*) of the Agaricomycotina [50]. The Woronin body and SPC clearly function in septal pore gating, however, the growth and development defects observed in Leashin [24] and SPC33 [42] mutants suggest additional roles. Understanding these should reveal additional links between these organelles and specific modes of multicellular organization.

Concluding remarks

The core functions of eukaryotic organelles are well understood, but relatively little is known about how organelles acquire taxa-specific functions to promote evolutionary radiation. Fungal pore-associated organelles evolved independently at least twice and appear to be key innovations. However, much remains to be done to validate this assertion and to understand their diversity and function (Box 1). In the pre-genomic era, compact genomes and powerful haploid genetics made cellular yeast ideal models for studying core eukaryotic function. In the future, the same attributes could make multicellular fungal phyla groups of choice for investigating the evolution of organelles and multicellular complexity.

Box 1. Outstanding questions

- How do the Woronin body and SPC modify and interact with core peroxisome and ER biogenesis functions? How is the abundance and quality of these derived organelles controlled?
- Like the Woronin body, the SPC is organized around a dense proteinaceous matrix (Figure 1c,d). Does the SPC dense-core self-assemble and promote organelle differentiation by influencing membrane composition as HEX does in the Woronin body biogenesis pathway (Figure 1f)?
- Key innovations can be defined as traits that promote ecological specialization and evolutionary radiation [46]. Are Woronin bodies and the SPC key innovations as suggested by their lifestyle-associated function? Future work aimed at determining whether these organelles acquired additional secondary functions during evolutionary radiation can help to further establish their credentials as key innovations.
- A number of functionally diverse proteins have been associated with Woronin bodies (Figure 4b). What is the functional significance of this localization?
- Leashin and SPC33 mutants display developmental defects that do not appear to be associated with pore occlusion – what role do these proteins play in the coordination of multicellular development?
- Pore-associated organelles also occur in other less-well-studied taxa [11,51,52]. Are these independent innovations, or are they related to the SPC or Woronin body?
- EM studies have shown that pore-associated organelles display systematic structural variation [10,11,52,53] – does this reflect random genetic drift or the evolution of specialized function?
- Looking more holistically at the biology of the fungi – what is the degree and quality of cellular innovation associated with a successful group such as the Pezizomycotina? Are there a few or many key innovations, and are these mostly functionally independent or highly integrated?

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