Report

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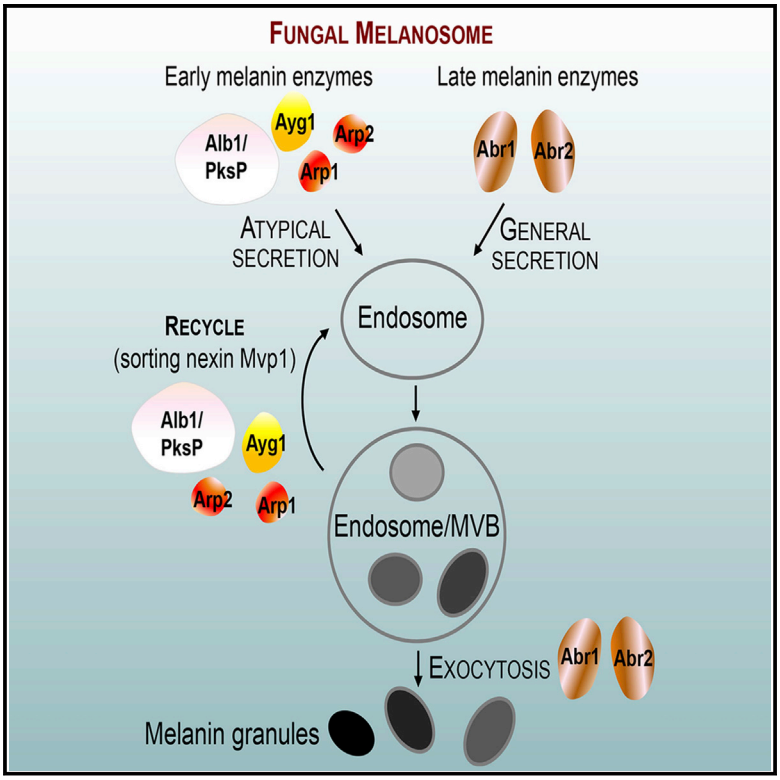
Subcellular Compartmentalization and Trafficking of the Biosynthetic Machinery for Fungal Melanin

Graphical Abstract



Highlights

- Endosomal trafficking is critical for melanization in fungi
- There is stage-specific subcellular localization of the melanin biosynthetic enzymes
- Early melanin enzymes have no secretion signal and are atypical secretory proteins
- There is a unified cellular principle for melanogenesis in mammals and fungi

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In Brief

Upadhyay et al. discovered that fungal melanin biosynthetic machinery is recruited to endosomes, although it is composed of largely atypical secretory proteins. Compartmentalization and trafficking through the endosomal system might be important cellular principles governing fungal secondary metabolism.

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Subcellular Compartmentalization and Trafficking of the Biosynthetic Machinery for Fungal Melanin

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SUMMARY

Protection by melanin depends on its subcellular location. Although most filamentous fungi synthesize melanin via a polyketide synthase pathway, where and how melanin biosynthesis occurs and how it is deposited as extracellular granules remain elusive. Using a forward genetic screen in the pathogen *Aspergillus fumigatus*, we find that mutations in an endosomal sorting nexin abolish melanin cell-wall deposition. We find that all enzymes involved in the early steps of melanin biosynthesis are recruited to endosomes through a non-conventional secretory pathway. In contrast, late melanin enzymes accumulate in the cell wall. Such subcellular compartmentalization of the melanin biosynthetic machinery occurs in both *A. fumigatus* and *A. nidulans*. Thus, fungal melanin biosynthesis appears to be initiated in endosomes with exocytosis leading to melanin extracellular deposition, much like the synthesis and trafficking of mammalian melanin in endosomally derived melanosomes.

INTRODUCTION

Melanins are resilient biopigments formed by oxidative polymerization of phenolic or indolic precursors. The contribution of melanization to species survival goes beyond colorization and UV protection. Melanins are used by the host to defend against microbes and conversely by microbes to invade their host. In insects, the formation of melanotic capsules is a key immune defense mechanism against microbial invasions (Lavine and Strand, 2002). In humans, melanins are involved in anti-microbial defense and immune modulation (Burkhart and Burkhart, 2005). However, melanins also protect pathogens from host immune responses or enable them to penetrate host barriers. The importance of melanin for microbial pathogenesis is illustrated in the human fungal pathogen *Aspergillus fumigatus*. This fungus causes a spectrum of diseases, including allergic bronchopulmonary aspergillosis, aspergilloma, chronic pulmonary aspergillosis, and the fatal invasive aspergillosis (Latgé and Steinbach, 2009). Melanin coated on spores plays a multifaceted protective role for *A. fumigatus* during infection: it promotes attachment to host tissues, helps evade host recognition by masking various pathogen-associated molecular patterns, scavenges reactive oxygen species (ROS) generated by phagocytes, prevents phagolysosome acidification, and inhibits macrophage apoptosis (Chotirmall et al., 2014).

The effectiveness of melanin host protection depends on its subcellular location. However, how melanin is synthesized and contained in fungal cells and how it gets deposited to the cell wall remain unclear. It is known that most filamentous fungi, including *A. fumigatus*, synthesize melanin via the polyketide pathway, which requires endogenous substrates (e.g., acetyl co-enzyme A [CoA]), a polyketide synthase (PKS), laccases, and sometimes additional modification enzymes. Because melanin, a negatively charged macromolecule, is found in the fungal cell wall as layers of globular particles (Eisenman et al., 2005; Hambleton et al., 2003; Walker et al., 2010), the cytoplasmic location of the synthases and substrates presents a conundrum for melanin's trafficking across the plasma membrane.

Here, a forward genetic screen in *A. fumigatus* uncovered an endosomal sorting mutant that lacks melanin deposition in the cell wall. We find compartmentalization of fungal melanin biosynthetic machinery to the endosomal system. This theme shows striking resemblance to melanosome biogenesis and trafficking in mammals, implicating a unified cellular principle governing melanization in eukaryotes. The importance of endosomes in compartmentalization and trafficking of the melanin biosynthetic machinery may apply to other fungal secondary metabolism pathways.

RESULTS

Mutations in the Endosomal Sorting Nexin Mvp1 Abolish Melanin Deposition to the Cell Wall

In *Aspergillus*, melanization occurs only during conidiation (Figure S1A). We previously performed a transfer DNA insertional mutagenesis screen in *A. fumigatus* to identify mutations that cause specific defects in conidial pigmentation (Jackson et al., 2009). Most color mutants harbored mutations in the melanin biosynthetic genes and showed conidial colors identical to the melanin gene deletion mutants. However, one insertional mutant, *mvp1*^{Tn} (referred to as #12, Jackson et al., 2009),

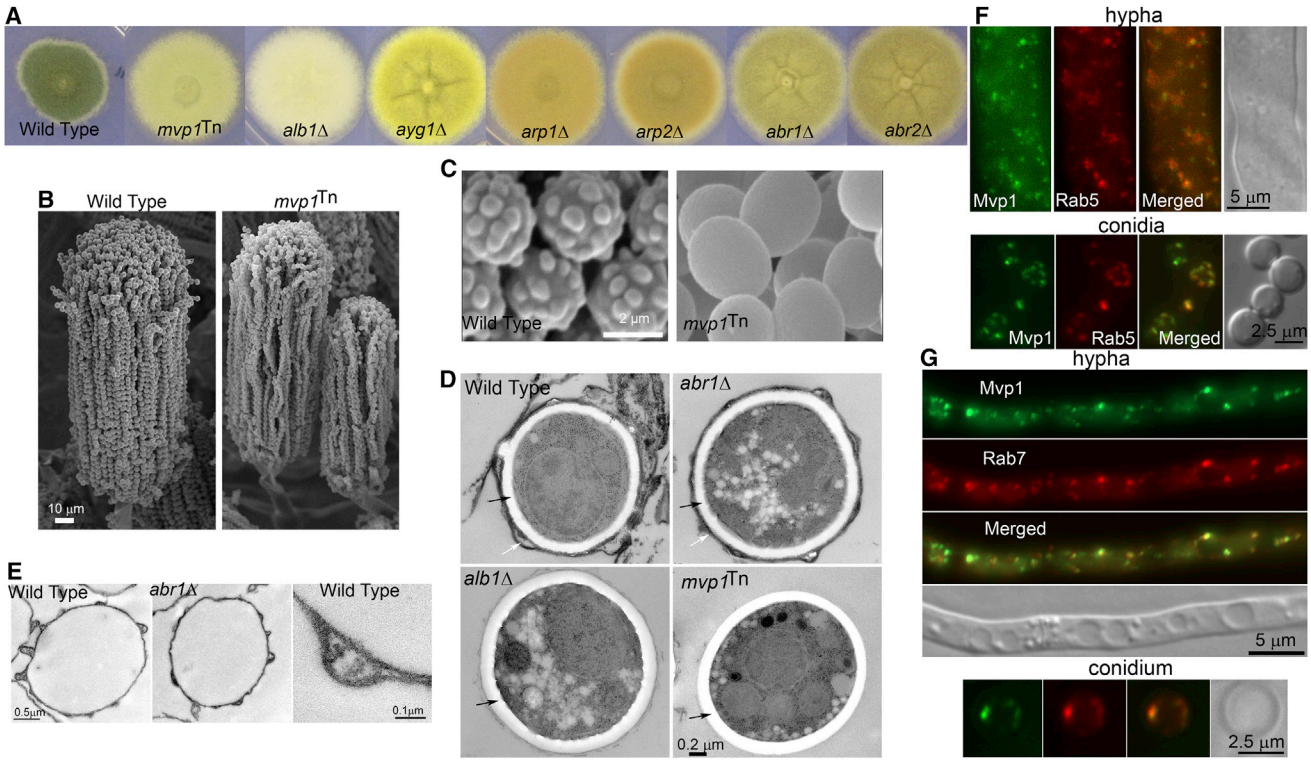


Figure 1. Mutations in the Endosomal Sorting Nexin Mvp1 Cause Lack of Melanin Deposition in the Cell Wall

(A) Colony images of WT, *mvp1*^{Tn}, and the melanin gene mutants (*alb1*Δ, *ayg1*Δ, *arp1*Δ, *arp2*Δ, *abr1*Δ, and *abr2*Δ).

(B) Scanning electron microscopy images of mature conidiophores of WT and the *mvp1*^{Tn} mutant.

(C) Scanning electron microscopy images of WT and *mvp1*^{Tn} conidia.

(D) TEM images of intact conidia of WT and the *alb1*Δ, *abr1*Δ, and *mvp1*^{Tn} mutants. White arrows point to the electron-dense melanin layer. Black arrows point to the electron-transparent cell-wall layer.

(E) TEM images of melanin ghosts extracted from WT and *abr1*Δ conidia.

(F) Localization of Mvp1 and the endosomal marker Rab5.

(G) Localization of Mvp1 and the endosomal marker Rab7.

See also Figure S1.

displayed a unique light conidial color (Figure 1A). In this *mvp1*^{Tn} mutant, no mutation was detected in the melanin gene cluster and all six melanin biosynthesis genes were induced during conidiation (Figure S1B), similar to wild type (WT) (Upadhyay et al., 2013). The results suggest that the pigmentation defect was not due to impaired expression of melanin biosynthetic genes.

It is known that deficiencies in conidial differentiation or maturation caused by mutations in AbaA or WetA also yield lighter color (Figure S1C) (Tao and Yu, 2011). However, *abaA*Δ produces aberrant conidiophores with no spores, while *wetA*Δ produces conidia with defective spore walls that results in characteristic cell lysis and deflation (Figure S1D) (Boylan et al., 1987; Tao and Yu, 2011). In contrast, the *mvp1*^{Tn} mutant developed normal conidiophores with long chains of conidia but no cell lysis (Figure 1B). Accordingly, no mutations were identified in the *abaA* or the *wetA* locus in this mutant.

Examination of the *mvp1*^{Tn} mutant by electron microscopy revealed a surprisingly smooth conidial surface (Figure 1C), similar to the albino mutant (*alb1*Δ or *pksP*Δ) that lacks melanin (Bayry et al., 2014; Tsai et al., 1998). Known mutants with lighter color, such as *wetA*Δ, still have an echinulated conidial surface (Figures S1C and S1D). This suggests that melanin is likely absent from the cell surface of the *mvp1*^{Tn} mutant. Consistently, after digestion with lysing enzymes and hot acid treatment to which melanin is resistant, the *mvp1*^{Tn} conidia showed no dark outer shell that was obvious in WT (Figure S1E), similar to the *ayg1*^{Tn} melanin mutant. We then extracted melanin ghosts from conidia of WT (positive control), the *ayg1*^{Tn} melanin mutant (negative control), and the *mvp1*^{Tn} mutant. No difference was observed in the dry biomass of the *mvp1*^{Tn} and *ayg1*^{Tn} melanin mutants. Thus, it appears that the *mvp1*^{Tn} mutant has little, if any, mature melanin in the cell wall.

Under a transmission electron microscope, melanin is the electron-dense layer outside of the electron-transparent cell wall in WT conidia, and it correlates with spikes that give rise to the echinulated conidial surface (Figures 1C and 1D). A similar electron-dense outer layer was observed in *abr1*Δ conidia, probably due to its ability to generate late melanin intermediates that can rectify cell-wall integrity (Bayry et al., 2014). By contrast, this electron-dense layer was absent from *alb1*Δ and *mvp1*^{Tn} conidia (Figure 1D). Furthermore, WT and *abr1*Δ conidia yielded sphere-shaped melanin ghosts that are similar in size to the original

conidia (Figure 1E). By contrast, the *alb1*Δ and *mvp1*^{Tn} mutants failed to yield discernible structures. Collectively, our data indicate that the *mvp1*^{Tn} mutant does not have mature melanin or late melanin intermediates in the cell wall.

We found that this insertional mutant had the Ti plasmid inserted into the *mvp1* gene that encodes a conserved endosomal sorting nexin (SNX8 in mammals) (Figures S1F and S1I). Deletion of *mvp1* in the model filamentous fungus *A. nidulans* likewise rendered a strain with a smooth conidial surface and defective pigmentation (Figure S1H). Introducing *A. nidulans* Mvp1-GFP into *A. nidulans mvp1*Δ mutant restored these phenotypes (Figure S1H). The *A. fumigatus* version of Mvp1-GFP also compensated for the loss of *mvp1* in *A. nidulans*, indicating cross-species conservation in Mvp1's function.

Like many other sorting nexins, Mvp1 contains a Bin-Amphiphysin-Rvs domain that can oligomerize and curve membranes, and a PHOX (or PX: phosphoinositide-binding domain) domain that recognizes phosphatidylinositol 3-phosphate, a lipid enriched in endosomal membranes (Figure S1F). In yeast and mammals, Mvp1 is broadly located in the endosomal system and participates in retrograde trafficking (Dyve et al., 2009; van Weering et al., 2012). In *A. fumigatus*, Mvp1-GFP was expressed at all developmental stages (Figure S1G). When co-expressed with the fluorescence-tagged early or late endosome marker Rab5 or Rab7, we found that Mvp1 largely matched the pattern of these endosomal markers (Figures 1F and 1G). Mvp1 and Rab5 or Rab7 also have distinct signals, as revealed by their fluorescence intensity profiles plotted along the cells (Figures S2A and S2B). Such a localization pattern of Mvp1 is consistent with the behaviors of the retromer SNX subcomplex observed in yeast and mammals, where a retromer could bulge from endosomes, forming tubule-like structures that are not decorated by Rab5 or Rab7 (Liu et al., 2012; Rojas et al., 2008). Some Mvp1-labeled puncta displayed rapid and long-distance movement (Movie S1), similar to Rab5-labeled early endosomes. The findings indicate that Mvp1 localizes broadly to the endosomal system in *Aspergillus*.

The Melanin Biosynthetic Machinery Shows a Stage-Specific Localization Pattern

The preceding evidence indicates that Mvp1 is important for *Aspergillus* melanization. Because export of certain cargo is deficient in *mvp1*Δ yeast cells (Chi et al., 2014), we hypothesize that one sorting nexin cargo in *Aspergillus* could be melanin biosynthetic enzymes. If true, melanin enzymes should be localized to endosomes.

In *A. fumigatus*, the polyketide-derived melanin is synthesized by six enzymes encoded by a gene cluster (Figure 2A) (Langfelder et al., 1998; Sugareva et al., 2006; Tsai et al., 1999, 2001). The foundation PKS enzyme Alb1 initiates melanin production from the substrates acetyl-CoA and malonyl-CoA. A series of subsequent enzymatic reactions carried out by Ayg1, Arp2, Arp1, and Arp2 leads to the production of vermelone, which is then oxidized and polymerized by the copper oxidase Abr1 and the laccase Abr2 to form mature melanin. The production of vermelone or the later melanin intermediate rectifies phenotypical defects caused by the lack of mature melanin, including cell surface structures and fungal virulence (Bayry et al., 2014). Consistently, we observed a normal conidial structure in *abr1*Δ (Figures 1D and 1E). Therefore, we classify enzymes that function before the vermelone production as early enzymes (Alb1, Ayg1, Arp1, and Arp2) and enzymes that function afterward as late enzymes (Abr1 and Abr2).

To our surprise, bioinformatic prediction indicates that only the late enzymes Abr1 and Abr2 are secretory proteins. All early enzymes are cytosolic proteins, consistent with their lack of a secretion signal or transmembrane domain (Figure 2A). Further analyses of additional species that synthesize melanin via the PKS pathway predicted that early enzymes are generally cytosolic while late enzymes are generally secretory (Figure 2A). This pattern is found across all fungal species examined, irrespective of their evolutionary distance (ascomycetes or basidiomycetes), the cluster organization of melanin genes, or the number of enzymes involved.

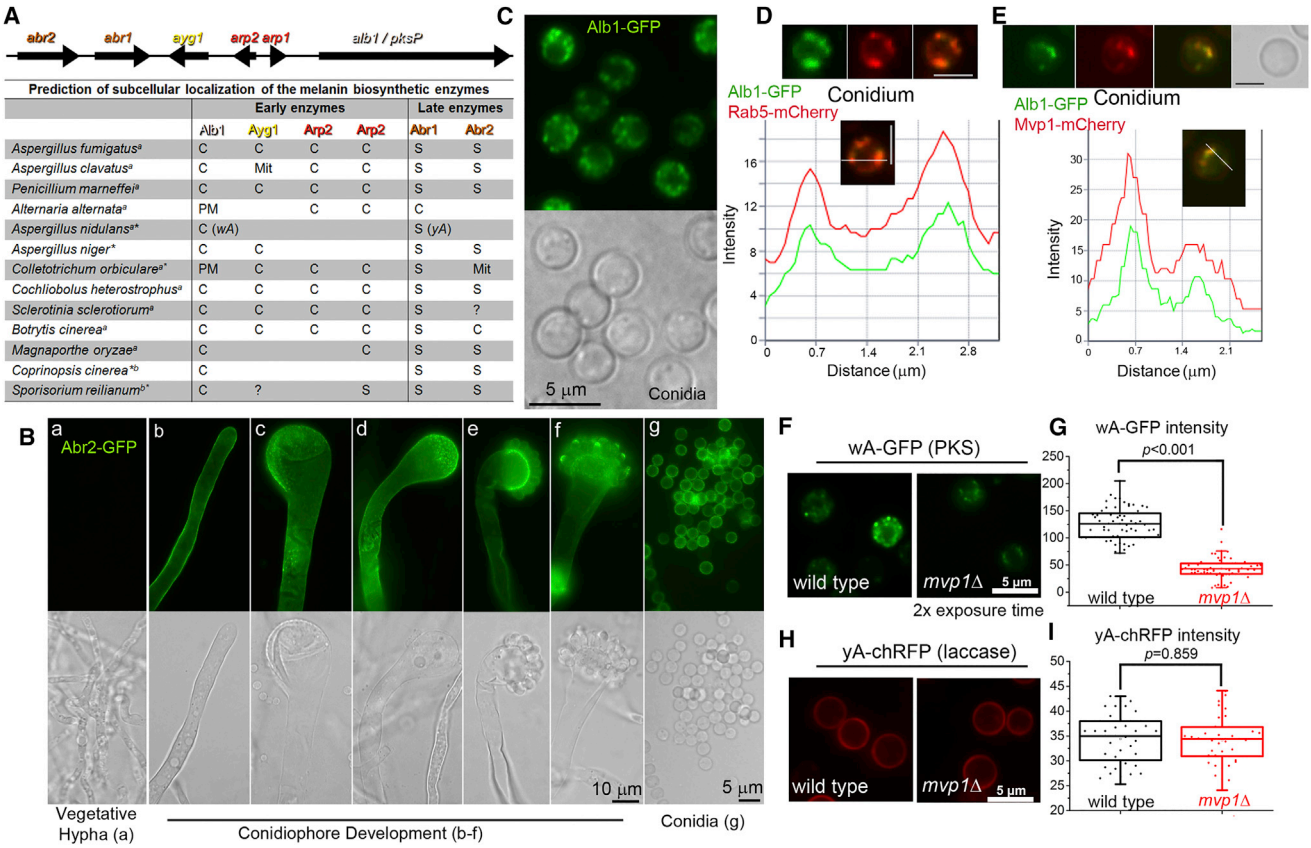
The predicted subcellular localization challenges our hypothesis that melanin enzymes traffic through endosomes. To investigate our hypothesis, we decided to verify experimentally the subcellular localization of the melanin biosynthetic machinery in *A. fumigatus*. We fluorescence-tagged all six melanin enzymes and used their native promoters to drive their expression. As expected, melanin enzymes were not expressed during vegetative hyphal growth but were produced in conidiophores during conidiation (Figure 2B) (Figure S2).

Both late enzymes, Abr1 (Upadhyay et al., 2013) and Abr2, as predicted, predominantly delineated the cell's outline, with some proteins localized to intracellular puncta (Figure 2B; Figure S2G). However, the PKS enzyme Alb1 did not show diffuse cytoplasmic localization as predicted. Rather, Alb1-GFP was localized to small puncta that resemble endosomes (Figure 2C). This multi-modular PKS enzyme (2,146 amino acids) with the fluorescence tag is functional based on its ability to restore *alb1*Δ's pigmentation defect (Figure S2C). Similarly, other early enzymes, despite their predicted cytosolic nature, were localized to small puncta (Figures S2D–S2F). Thus, the melanin enzymes displayed two distinct localization patterns: the early enzymes are located to intracellular punctate structures, while the late enzymes are prominently located to the cell periphery.

To examine whether the two localization patterns apply to other fungi, we tagged two known melanin enzymes in *A. nidulans*. The evolutionary distance between *A. fumigatus* and *A. nidulans* is comparable to that between mammals and fish. Unlike *A. fumigatus*, which uses six enzymes encoded by a gene cluster, *A. nidulans* uses two unlinked enzymes for melanization: the PKS wA and the laccase yA (Figure 2A) (Aramayo and Timberlake, 1990; Tsai et al., 1999). Nonetheless, we found that the early enzyme wA was localized to intracellular puncta (Figure 2F) and the late enzyme yA mostly outlined the cells (Figure 2H). Thus, it appears that irrespective of the number of enzymes involved in melanin biosynthesis or the cluster arrangement, the early enzymes are in intracellular punctate structures while the late enzymes are secreted to the cell periphery.

Early Melanin Biosynthetic Enzymes Co-localized with Endosome Markers

To examine whether the intracellular puncta highlighted by the early enzymes are endosomes, we introduced the fluorescently



Prediction of subcellular localization of the melanin biosynthetic enzymes

	Early enzymes				Late enzymes	
	Alb1	Ayg1	Arp2	Arp2	Abr1	Abr2
Aspergillus fumigatus[a]	C	C	C	C	S	S
Aspergillus clavatus[a]	C	Mit	C	C	S	S
Penicillium marneffei[a]	C	C	C	C	S	S
Alternaria alternata[a]	PM		C	C	C	
Aspergillus nidulans[a]*	C (*wA*)				S (*yA*)	
*Aspergillus niger**	C	C			S	S
Colletotrichum orbiculare[a]*	PM	C	C	C	S	Mit
Cochliobolus heterostrophus[a]	C	C	C	C	S	S
Sclerotinia sclerotiorum[a]	C	C	C	C	S	?
Botrytis cinerea[a]	C	C	C	C	S	C
Magnaporthe oryzae[a]	C			C	S	S
Coprinopsis cinerea[b]*	C				S	S
Sporisorium reilianum[b]*	C	?		S	S	S

Figure 2. Distinct Localization Patterns for the Early and Late Melanin Enzymes

(A) The melanin gene cluster in *A. fumigatus* and the predicted localization of melanin enzymes in diverse fungal species. S, secreted protein; C, cytosolic protein; PM, plasma membrane; Mit, mitochondria; [a], ascomycetes; [b], basidiomycetes; *, melanin genes not arranged in a cluster; ?, the presence of an ortholog is uncertain.

(B) The expression and localization of Abr2 during development. (a) Vegetative hypha, (b) young stalk, (c and d) mature stalk, (e and f) young conidiophore, and (g) conidia.

(C) Localization of Alb1 in conidia.

(D) Localization of Alb1-GFP and the endosomal marker Rab5-mCherry. A fluorescence intensity plot along a cellular axis is indicated with a white line.

(E) Localization of Alb1-GFP and Mvp1-mCherry. A fluorescence intensity plot shows co-localization.

(D and E) Scale bars represent 2.5 μm.

(F and G) Localization (F) and fluorescence intensity (G) of the early melanin enzyme wA-GFP in WT and *mvp1*Δ in *A. nidulans*.

(H and I) Localization (H) and fluorescence intensity (I) of the late melanin enzyme yA-chRFP in WT and *mvp1*Δ.

See also Figure S2.

labeled endosomal marker Rab5 into these strains. Alb1 localized to the same structures as Rab5 (Figure 2D). Similar co-localization with Rab5 was observed for two other early enzymes tested (Arp1 and Arp2) (Figures S2I and S2J). Moreover, these early enzymes largely matched the localization of the endosomal sorting nexin Mvp1 (Figure 2E; Figure S2H).

Because Mvp1 is known to be involved in retrograde trafficking, we examined the impact of *mvp1* disruption on the melanin enzymes. For this purpose, we compared the localization and intensity of the early enzyme wA-GFP and the late enzyme yA-RFP in WT and *mvp1*Δ in *A. nidulans*. In *mvp1*Δ, wA-GFP was still localized to puncta, but the fluorescence intensity was much lower than that in WT (Figures 2F and 2G). By contrast, there was no apparent difference in yA-RFP localization or fluorescence intensity with or without Mvp1 (Figures 2H and 2I). The observations indicate that Mvp1 helps maintain the protein level of the early melanin enzyme, possibly by recycling the proteins for repetitive usage. We reasoned that if early melanin enzymes are drastically reduced in the *mvp1* mutant, the level of early melanin intermediates would accordingly be decreased. To test this hypothesis, we treated WT and the *ayg1* and *mvp1*^{Tn} mutants with tricyclazole. Tricyclazole inhibits Arp2 and causes accumulation of the shunt product flaviolin, derived from the intermediate produced by Ayg1 (Tsai et al., 2001). As expected, tricyclazole treatment altered the *mvp1*^{Tn} conidial color only slightly (Figure S2O) and resulted in no detectable accumulation of the predicted flaviolin by thin layer chromatography (Figure S2P). The results are consistent with the predicted low level of melanin intermediates in *mvp1* mutants.

Copper is an obligate co-factor for melanin enzymes (Chang, 2009). In *A. fumigatus*, the P-type Cu-transporter CtpA is required for melanization under copper-limiting conditions

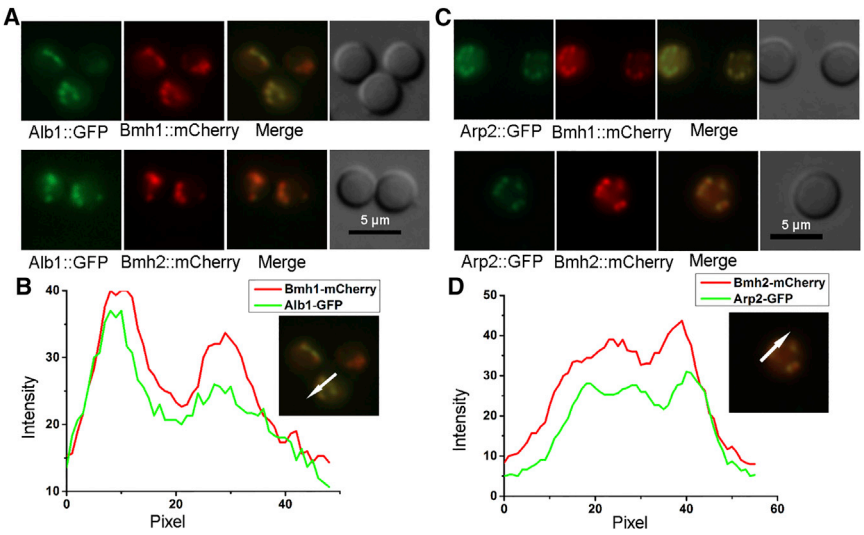


Figure 3. Co-localization of Two 14-3-3 Isoforms, Bmh1 and Bmh2, with Melanin Enzymes Alb1 and Arp2

(A) Images of Alb1-GFP with either Bmh1-mCherry (top) or Bmh2-mCherry (bottom) in conidia.

(B) The fluorescence intensity profiles of Alb1-GFP and Bmh1-mCherry plotted against the conidial cellular axis in the direction shown in the images at the right.

(C) Images of Arp2-GFP with either Bmh1-mCherry (top) or Bmh2-mCherry (bottom) in conidia.

(D) The fluorescence intensity profiles of Arp2-GFP and Bmh2-mCherry plotted against the conidial cellular axis in the direction shown in the images at the right.

(Figure S2K) (Upadhyay et al., 2013). P-type transporters typically deliver copper from the cytosol to the lumen of a secretory compartment (Banci et al., 2010). Consistently, CtpA was detected in small vesicles when copper was limiting and in vacuoles when copper was excessive (Figure S2L). Under normal growth conditions, the distribution of CtpA in the cell largely overlapped with Mvp1 and the early enzyme Alb1 (Figure S2M). Collectively, the findings suggest that early melanin enzymes and CtpA (to supply the copper co-factor) are recruited to endosomes to facilitate melanogenesis during conidiation.

Early Melanin Enzymes Showed Co-localization with MVB Markers

Given that none of the early melanin enzymes have a secretion signal or a transmembrane domain, how they become associated with endosomes and whether they function in the cytosolic side or in the lumen of the organelle are unknown. Because previous observations in other fungi indicate melanin or melanin intermediates in intracellular and extracellular vesicles, and mature melanin as granules in the cell wall (Alviano et al., 1991; Eisenman et al., 2005; Hambleton et al., 2003; Walker et al., 2010), we conjectured that melanin enzymes likely function in the lumen of the endomembrane system, which could be achieved through membrane invagination, as in the formation of multivesicular bodies (MVBs).

A marker for MVBs and extracellular vesicles in the fungus *Cryptococcus neoformans* is 14-3-3 (Li et al., 2015). The 14-3-3 proteins are also found in extracellular vesicles in mammals (Pisitkun et al., 2004). *A. fumigatus* has two 14-3-3 isoforms, Bmh1 and Bmh2. We fluorescently tagged both Bmh1 and Bmh2 and compared their subcellular localization with early melanin enzymes Alb1 and Arp2. Both 14-3-3 isoforms co-localized with the two melanin enzymes tested (Figure 3), suggesting that endosome-derived MVBs might constitute an important cellular compartment for these melanin enzymes. Consistent with this idea, the activity of laccase, the enzyme responsible for *Cryptococcus* melanization, is drastically reduced in a strain with a low level of the 14-3-3 protein (Li et al., 2015).

Late Melanin Enzymes Are Secreted and Accumulated in the Cell Wall

Different from early enzymes, both late enzymes, Abr1 and Abr2, are mostly located to the cell periphery (Figure 2B; Figure S2G). To ascertain whether they are accumulated in the plasma membrane or the cell wall, we designed two approaches to separate the cell wall from cytoplasm. We first tested the classic plasmolysis approach that compresses cytoplasm using the *A. nidulans* strain with GFP-SsoA, a known plasma membrane t-SNARE (target- soluble NSF attachment protein receptor) (Figure S3A) (Taheri-Talesh et al., 2008). As expected, the GFP-SsoA signal stayed with cytoplasm that was separated from the cell wall during plasmolysis (Figure S3B). By contrast, Abr1-GFP was mostly retained in the cell wall, while intracellular Mvp1-mCherry was compressed with cytoplasm away from the cell wall (Figure 4A). Abr2 was also primarily retained in the cell wall during plasmolysis, similar to Abr1 (Figure 4C). In *abr1*Δ, Abr2-GFP was still secreted, but the fluorescence intensity was much reduced (Figures 4D and 4E). This suggests that Abr1 is needed for Abr2's stability but not its secretion. In the second approach, we mechanically severed the cell. Upon wounding, septum pores will be sealed to prevent excessive cytoplasmic loss in the neighboring cell. As expected, GFP-SsoA was retained with the cytoplasm only in the hyphal cell adjacent to the damaged cell (Figure S3C). Similarly, intracellular Mvp1-mCherry was absent from the damaged conidiophore stalk cell but it was present in the neighboring intact cells on the conidiophore head (Figure 4B) due to the protection by sealed septa. By contrast, Abr1 was present in both the adjacent intact cells and the severed stalk cell (Figure 4B), indicating that Abr1 was retained in the cell wall of the damaged cell. Taken together, the findings demonstrate that Abr1 and Abr2 accumulate in the cell wall.

DISCUSSION

Our findings of stage-specific subcellular compartmentalization of melanin biosynthetic machinery and previous observations of melanin in intracellular and extracellular vesicles lead us to propose that melanin biosynthesis and trafficking occur in the endomembrane system and that the late enzymes and mature

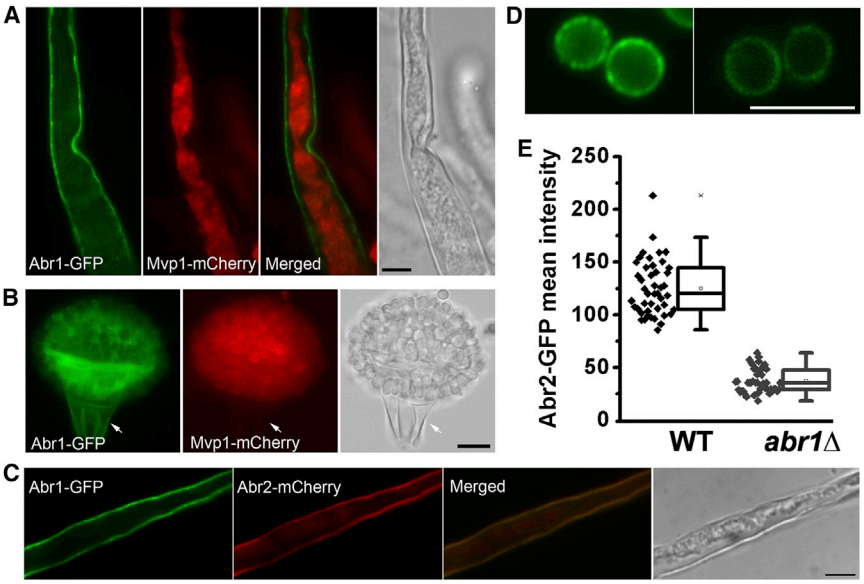


Figure 4. The Late Enzymes Accumulate in the Cell Wall

(A) Plasmolysis reveals the cell-wall localization of Abr1 and intracellular localization of Mvp1.

(B) Mechanic severing reveals Abr1's association with the cell wall in the damaged stalk cell. Intracellular Mvp1 is retained in the intact conidia but is lost in the severed stalk cell.

(C) Cells expressing Abr1-GFP and Abr2-mCherry were treated for plasmolysis. The separation of cytoplasm from the cell wall (differential interference contrast) reveals the localization of both Abr2 and Abr1 in the cell wall. Scale bar represents 5 μm.

(D and E) Localization (D) and fluorescence intensity (E) of Abr2-GFP in WT and *abr1*Δ ($p < 0.001$). Scale bars represent 5 μm.

See also Figure S3.

melanin are exported through exocytosis. In many aspects, endosomes with melanin produced by *Aspergillus* during conidiation resemble the well-recognized tissue- and physiologically specific lysosome-related organelles (LROs) that are derived from the endosomal system in higher eukaryotes. One prominent type of LRO is melanosomes that confer color to the skin, eyes, and hair. In mammals, melanin is synthesized in melanosomes by melanocytes, which are transferred to neighboring keratinocytes, likely via shedding or exocytosis by melanocytes, followed by internalization by keratinocytes (Wu and Hammer, 2014). Thus, intracellular transport and intercellular transfer of melanosomes are as important for pigmentation as the biosynthesis of melanin in mammals.

In both *Aspergillus* and mammals, pigmentation requires three elements: melanin biosynthetic machinery, melanosome structure, and intracellular and extracellular trafficking. Mutations in any of the three aspects result in diseases such as hypopigmentation or albinism. Examples in mammals include defects in targeting the melanin biosynthetic machinery to melanosomes (enzymes TYR/TYRP1 and the copper transporter ATP7A) (Setty et al., 2008; Sitaram and Marks, 2012) or in melanosome trafficking (Barral and Seabra, 2004). The human melanin enzymes TYR/TYRP1 are both classical secreted proteins, similar to some yeasts that only use classically secreted laccases to polymerize exogenous precursors for melanization (Eisenman et al., 2005). By contrast, *Aspergillus* early melanin enzymes have no secretion signal and are thus recruited to the endosome via a non-conventional secretion pathway. In both systems, having membrane-delimited organelles for melanization could confer several advantages: it concentrates substrates or precursors and multiple enzymes that need to function sequentially (metabolic channeling), sequestration of toxic intermediates minimizes potential damage to other cytoplasmic machineries, and the endosomal system provides mobility and effective exportation of the negatively charged macromolecules across the plasma membrane. Thus, despite the diversity in enzymes and substrates involved in making melanin, the remarkable resemblance between fungal and mammalian melanosomes suggests the conserved cellular principles evolved for melanization in eukaryotes.

Melanins are unique in that they are macropolymers that act as a structural component. Besides melanin, fungal PKS pathways produce various small polyketides that have great economic and health impacts. One well-characterized polyketide, aflatoxin, is synthesized and trafficked in aflatoxisomes (Chanda et al., 2010), which were established as endosomes (Ehrlich et al., 2012). Aflatoxin and its intermediate are presumably exported in endosome-derived MVBs, as evidenced by the detection of these compounds in discrete round patches on the intact cell surface (Chanda et al., 2010). Certain enzymes of some other PKS or nonribosomal peptide synthetase (NRPS) pathways are known to localize to vesicles, including some involved in synthesizing penicillin (Müller et al., 1992), cyclosporin (Hoppert et al., 2001), or trichothecene (Menke et al., 2013). The first complete gene cluster to be characterized for their enzymes' subcellular location is the fumiquinazoline cluster, where all four enzymes are localized by GFP tagging using overexpressing strains (Lim et al., 2014). The enzyme involved in the last step of fumiquinazoline production is localized to the cell wall (Lim et al., 2014), similar to the late melanin enzymes that we reported here. It is conceivable that compartmentalization of all or some biosynthetic enzymes is a common mechanism to control the biogenesis and trafficking of various secondary metabolites (Keller, 2015; Kistler and Broz, 2015).

Compartmentalization in secretory vesicles might apply to other biosynthetic machineries. For example, chitin, the defining fungal cell-wall component, is synthesized in membrane-delimited structures called chitosomes (Bartnicki-Garcia, 2006). The polysaccharide capsule materials in *Cryptococcus* are found in extracellular vesicles derived from MVBs (Rodrigues et al., 2008), and they are likely both synthesized and trafficked in endosomes. Thus, using vesicles might be a unified cellular principle to direct biosynthesis of macromolecules in a defined

area and to provide the mobility for enzymes and secreted products.

The finding of endosomal or MVB localization of all four early melanin enzymes was unexpected, given that none of them are considered conventional secretory proteins. The discovery provides an excellent example of trafficking to endosomes or MVBs of biosynthetic machinery that is composed of largely atypical secretory proteins. The requirement of a classic endosomal sorting nexin suggests that non-canonical secretion pathway uses the conventional endomembrane system. Thus, the critical step for atypical protein secretion might be recruitment.

These discoveries raise many questions that are not limited to fungal biology. LROs are possibly used to synthesize and/or sort cargo by diverse organisms for various functions, as in the delivery virulence factors by fungi (Yi and Valent, 2013), the facilitation of host entry by trypanosomes (Andrews, 2002), or the generation or delivery of cytolytic granules by cytotoxic T cells and natural killer cells (Bonifacino, 2004). Do other LROs share common characteristics with melanosomes? Studying fungal melanosomes could yield powerful insights into such questions that are fundamental to our understanding of eukaryotic biology.

EXPERIMENTAL PROCEDURES

Strains, Gene Expression, and Western Blot

Strains and primers are listed in Tables S1 and S2, respectively. Details of gene deletion, transcript analyses, protein tagging, sequencing, prediction of subcellular localization, protein extraction, and western blotting can be found in the Supplemental Experimental Procedures.

Microscopy

The expression of all fluorescence-tagged proteins was driven by their own promoters. Details for their construction, the measurement of fluorescence intensity, and fluorescence intensity profiling can be found in the Supplemental Experimental Procedures. Procedures of sample preparation and analyses for scanning and transmission electron microscopy (TEM) are also included in the Supplemental Information.

Plasmolysis and Mechanic Severing

The aerial conidiophore structures of *A. fumigatus* are highly resistant to osmolytes such as glycerol or sorbitol. Thus, we used ethanol to render the membrane leaky to small molecules and the cytoplasm compressed under such a condition. Severing the conidiophore stalk or hypha by forceps caused loss of cytoplasm, rendering a ghost cell with only the cell wall remaining. Other cells connected to the ghost cell remained intact because of protection by septa. In Figure 4B, the cell-wall signal from Abr1 from the conidiophore that included the stalk cell, phialides, and conidia appeared overwhelming due to the clustering of these many cells. Images of individual conidia or the stalk clearly showed cell-wall localization of Abr1 and Abr2.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, two tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.059.

AUTHOR CONTRIBUTIONS

S.U., X.X., and X.L. conceived and designed the experiments; S.U., X.X., J.C.J., D.L., and X.L. performed the experiments; S.U., X.X., J.C.J., D.L., R.W.R., and X.L. analyzed the data; and S.U., X.X., D.L., and X.L. wrote the paper.

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