Aspergillus nidulans biofilm formation modifies cellular architecture and enables light-activated autophagy

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ABSTRACT After growing on surfaces, including those of medical and industrial importance, fungal biofilms self-generate internal microenvironments. We previously reported that gaseous microenvironments around founder Aspergillus nidulans cells change during biofilm formation causing microtubules to disassemble under control of the hypoxic transcription factor SrbA. Here we investigate if biofilm formation might also promote changes to structures involved in exocytosis and endocytosis. During biofilm formation, the endoplasmic reticulum (ER) remained intact but ER exit sites and the Golgi apparatus were modified as were endocytic actin patches. The biofilm-driven changes required the SrbA hypoxic transcription factor and could be triggered by nitric oxide, further implicating gaseous regulation of biofilm cellular architecture. By tracking green fluorescent protein (GFP)-Atg8 dynamics, biofilm founder cells were also observed to undergo autophagy. Most notably, biofilm cells that had undergone autophagy were triggered into further autophagy by spinning disk confocal light. Our findings indicate that fungal biofilm formation modifies the secretory and endocytic apparatus and show that biofilm cells can also undergo autophagy that is reactivated by light. The findings provide new insights into the changes occurring in fungal biofilm cell biology that potentially impact their unique characteristics, including antifungal drug resistance.

INTRODUCTION In nature, many cells exist within organs or colonies in a viable but static nongrowing state and generate their own unique microenvironments. The microenvironment of cells plays important roles in many biological processes including tumorigenesis (Wang et al., 2017) and drug resistance of microbial pathogens. With regards to microbial infections, the physiological state of static cells impacts how pathogens respond to drug treatment. For example, fungal biofilm cells generate their own microenvironments and resistance to antifungal treatments (Desai et al., 2014; Kaur and Singh, 2014; Kowalski et al., 2020). On the more positive side, solid state fungal biofilms can be the preferred mode of growth in fungal driven fermentations, as well as production of enzymes, organic acids, and other bioactive compounds (Gutierrez-Correa et al., 2012; Park et al., 2017). However, the biological changes occurring within fungal biofilm cells which distinguish them from unattached planktonically growing cells is not well understood. There is therefore considerable interest in understanding how the physiology and cell biology of forming biofilm cells get modified (Gonzalez-Ramirez et al., 2016) and how this might be leveraged to help remediate negative fungal biofilm characteristics, such as during food spoilage and infections. Similarly, further understanding of changes occurring within fungal biofilms could help enhance their industrial applications (Villena and Gutierrez-Correa, 2007).

Biofilm formation starts with the attachment of initiator cells to a surface (Ramage et al., 2011; Gonzalez-Ramirez et al., 2016). For Aspergillus nidulans biofilm formation, growth is initiated using asexual spores called conidia. Germinating conidia have a tenacious...
capacity to attach to surfaces including glass. For example, during liquid growth in unsiliconized glass flasks, germinating conidia will attach and grow on the sides of conical flasks even when the flasks are rotated at a high speed. Growth on a surface as a biofilm is likely the preferred mode of growth for A. nidulans and many other Aspergillus species (Gravelat et al., 2010). To track the cell biology of founder biofilm cells, they are grown in dishes containing a coverslip at their bottom. Germinating conidia attach and grow on the coverslip and can be observed during biofilm formation using an inverted spinning disk confocal microscope system (Shukla et al., 2017). Founder tip cells initially avoid each other as they grow but then some stop growing, getting trapped at the base of the forming biofilm. During biofilm development, microtubules (MTs) then get depolymerized within the founder cells in a manner that was reversible by gaseous exchange above the culture dish (Shukla et al., 2017). MT disassembly failed to occur in strains lacking the hypoxic transcription factor SrbA, indicating that changing gaseous microenvironments play a role in regulating biofilm cell biology. SrbA (Willger et al., 2008; Bat-Ochir et al., 2016) is the Aspergillus orthologue of the Schizosaccharomyces pombe sterol regulatory element-binding protein 1 (Sre1), a transcription factor (Hughes et al., 2005) required for adaptation to hypoxia. As seen in other fungi, deletion of srbA in A. nidulans does not affect growth on agar plates under normoxia but inhibits growth under hypoxic growth conditions (Bat-Ochir et al., 2016). Collectively the findings indicate that the cell biology of founder biofilm cells is modified in a process involving self-generated hypoxia caused by metabolic O₂ depletion outcompeting its rate of replacement via diffusion, and SrbA regulation (Shukla et al., 2017).

In the current study, we aimed to ask if the cell biology of biofilm founder cells is changed beyond MT depolymerization and if other more widespread systemic changes in subcellular architecture might occur under control of SrbA. The primary function of cytoplasmic MTs is to transport membranous cargoes, including early endosomes and post-Golgi vesicles (Abenza et al., 2009; Pantazopoulou et al., 2014). Therefore, the impairment of cargo transport resulting from MT-depolymerization during biofilm formation could be coordinated with functional or structural changes in upstream and downstream steps of the membrane trafficking pathway. Furthermore, we reasoned that during biofilm formation the growth arrest of founder tip cells might be accompanied by blockages or flux-changes in secretion and endocytosis, two processes whose coordinated action accomplishes polarized tip growth (Riquelme et al., 2018). To test these ideas, we first investigated the dynamics of the central components of the membrane trafficking pathway and found that while the peripheral endoplasmic reticulum (ER) remained intact, the transitional ER, the early and late Golgi cisternae, and endocytic actin patches were dramatically modified. Also, during these studies we discovered that autophagy, tracked by following green fluorescent protein (GFP)-Atg8 localization, was reactivated during spinning disk confocal imaging of biofilm founder cells and we discuss the potential molecular basis for this effect.

RESULTS

The ER remains intact during biofilm formation
To investigate if the protein secretory apparatus might change during biofilm formation, we first imaged the ER using Sec63-GFP, a marker previously used to monitor ER dynamics in A. nidulans (Markina-Inarraiaegui et al., 2013). We also tracked EB1-mCherry comets which locate at the ends of growing MTs. EB1-mCherry undergoes dispersal from comets during early biofilm formation after MTs disassemble and so can be used as a marker for biofilm formation (Shukla et al., 2017). Cells were imaged after 24 h of growth, representing an early stage of biofilm formation prior to MT disassembly and EB1-mCherry dispersal (Figure 1A, Day 1). Cells were additionally imaged after 3 d of growth by which time biofilm maturation had promoted MT disassembly and EB1-mCherry dispersal (Figure 1A, Day 3). As previously reported (Markina-Inarraiaegui et al., 2013), Sec63-GFP locates throughout the ER including around nuclei at the outer nuclear envelope, as well as in cytoplasmic ER strands and tubules, many of which were plasma membrane associated (Figure 1A, Day 1). After biofilm formation, no obvious alterations in ER structures were observed with, or without, SrbA function (Figure 1, A and B).

Without SrbA cells can accumulate autofluorescent species during biofilm formation
During biofilm formation of ΔsrbA cells, in addition to EB1-mCherry comets, other red signals appeared in vacuolelike structures. These additional signals were absent from WT biofilm cells that were similarly tagged. To distinguish if the additional red signal in ΔsrbA biofilm cells was from autofluorescence specific to ΔsrbA cells, we imaged WT and ΔsrbA cells during biofilm formation which lacked

![Figure 1: The ER appears unchanged during biofilm formation. (A) The ER marker Sec63-GFP and the MT +End-binding protein EB1-mCherry (strain SO1812) before (Day 1) and during biofilm formation (Day 3). EB1-mCherry undergoes dispersal from comets during biofilm formation but the Sec63-GFP ER pattern remained unchanged. (B) Same as for A but in a strain with srbA deleted (srbAΔ, strain SO1810). We observed some vacuolelike signal in the red channel of some srbAΔ cells which represents autofluorescence specific to srbAΔ cells; see Supplemental Figure S1. (C) The ER marker Sec63-GFP (strain SO1812) before and 15 min after treatment with the NO donor NOC-12 as indicated. Bar, 10 μm.](https://example.com/figure1.png)
fluorescent protein markers (Supplemental Figure S1). In wild-type cells, no red autofluorescence was apparent before or during biofilm formation. However, in ΔsrbA cells, red autofluorescence became apparent during biofilm maturation (Supplemental Figure S1) within vacuole-like structures (Veses et al., 2008). While autofluorescence can be a confounding issue during microscopy, it is possible to discern differences between autofluorescence and tagged EB1 because of their distinctive patterns and movements.

**ER exit sites are modified in an SrbA-dependent manner during biofilm formation**

We next investigated the behavior of ER exit sites (ERESs) during biofilm formation by visualizing the COPII coat component Sec23 (Rossanese et al., 1999; Budnik and Stephens, 2009) C-terminally tagged with GFP. Sec23-GFP has previously been employed to track ERESs in A. nidulans (Pantazopoulou and Penalva, 2009) and shown to locate at many small foci with an increased density toward cell tips (Pantazopoulou and Penalva, 2009), a pattern we also observed (Figure 2A, Day1). During the initial growth phase of biofilm formation, the founder cells contained many ERES foci and EB1-mCherry located to comets (Figure 2A, Day 1). During biofilm formation, EB1-mCherry dispersed from comets and Sec23-GFP was observed to locate to fewer, larger foci (Figure 2A, Day 3).

To ask if the Sec23 ERES modifications were dependent on the hypoxic transcription factor SrbA, the experiment was repeated under the same biofilm forming conditions but in a ΔsrbA strain lacking SrbA. Without SrbA function, EB1-mCherry remained at comets during biofilm formation as previously noted (Shukla et al., 2017), and Sec23-GFP also remained unchanged, continuing to locate to numerous ERES foci (Figure 2B). This shows that, like MT disassembly, modification of ERESs during biofilm formation is dependent on SrbA.

**Nitric oxide treatment phenocopied the effects of biofilm formation on ERES and EB1**

Treatment of A. nidulans cells with hydrogen sulfide (H₂S) caused MT disassembly and dispersal of EB1 from comets, as occurs in biofilm cells (Shukla et al., 2017). It was suggested these effects could be caused by H₂S inhibiting cytochrome c oxidase and respiration (Mustafa et al., 2009). We therefore tested if nitric oxide (NO), which also inhibits cytochrome c oxidase, might have similar effects and cause the changes to ERESs as seen during biofilm formation. NOC-12 [N-Ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine] is a NO donor with a half-life, at pH 7.4, of over 5 h. Notably, the ER pattern of Sec63-GFP was not modified during biofilm formation (Figure 1, A and B) nor did NO treatment notably modify ER structure of growing nonbiofilm cells (Figure 1C). In contrast, treatment of growing nonbiofilm cells with NOC-12 for 15 min caused the conversion of Sec23-GFP from small ERES foci to fewer larger foci (Figure 2C, +NOC-12), a transition mirroring what happens to ERES foci during biofilm formation (Figure 2A). We conclude that NO promotes similar effects on Sec23-GFP as occurs during biofilm formation.

**The early Golgi marker GrhA is modified in an SrbA-dependent manner during biofilm formation and after NO treatment**

The A. nidulans orthologue of Saccharomyces cerevisiae Grh1p (termed GrhA) endogenously C-terminally tagged with GFP acts as an early Golgi marker in A. nidulans (Pantazopoulou and Penalva, 2009). In yeast, Grh1p targets the coiled-coil tether Bug1 to the early Golgi and interacts with the COPII Sec23/24 subcomplex (Behnia et al., 2007). In nonbiofilm A. nidulans cells, GrhA-GFP localizes to foci that are less abundant than those of the ERES marker Sec23-GFP (Pantazopoulou and Penalva, 2009) a pattern we also observed (Figure 3A, Day1). During biofilm formation GrhA localizes to fewer, larger foci (Figure 3A, Day 3) and, similar to Sec23, this transition was dependent on SrbA (Figure 3B). The transition of GrhA-GFP to fewer larger foci, as seen during biofilm formation, was recapitulated by NO treatment of growing nonbiofilm cells (Figure 3C).

**Golgi organization is modified in an SrbA-dependent manner during biofilm formation and after NO treatment**

A trans-Golgi-specific reporter construct termed gpdA::PH::mRFP-Phos, which expresses the pleckstrin homology domain of the human oxysterol binding protein (PH<sup>osBP</sup>) fused to mRFP, was developed as a Golgi marker in A. nidulans (Pantazopoulou and Penalva, 2009). As previously observed, mRFP-PhosBP locates in a tip-polarized pattern of foci representing trans-Golgi cisternae (Pantazopoulou and Penalva, 2009; Figure 4A, Day 1). Notably, the trans-Golgi was seen to undergo modifications during biofilm formation with mRFP-PhosBP then locating throughout the cytoplasm (Figure 4A, Day 3).
Endocytic internalization actin patches are modified in an SrbA-dependent manner during biofilm formation

Given that the protein secretory apparatus is modified during biofilm formation (Figures 1–4), we asked if endocytosis might also be affected. We explored how the orthologue of the endocytic actin patch marker Abp1 of *S. cerevisiae*, termed AbpA in *A. nidulans* (Araujo-Bazan et al., 2008), might change during biofilm formation. Endogenously mRFP tagged AbpA locates to patches at the cell periphery as a marker for endocytic actin patches, with some patches forming a subapical cell tip ringlike pattern (Araujo-Bazan et al., 2008; Figure 5A, Day 1). During biofilm development, AbpA-mRFP locations are modified such that larger, less abundant foci appear while the small peripheral patches were no longer apparent (Figure 5A, Day 3). Notably, it has been shown that treatment of cells to lower ATP using 10 mM sodium azide plus 10 mM sodium fluoride treatment also caused depolarization and clustering of AbpA patches into larger structures (Araujo-Bazan et al., 2008).

Most of the changes occurring to AbpA-mRFP during biofilm formation failed to occur in strains lacking SrbA function, although the tip-centric location of the peripheral foci appeared diminished (Figure 5B, Day 3).

**Autophagy is activated during biofilm maturation**

Autophagy enables cells to utilize cytoplasmic constituents and those sequestered in organelles via their breakdown in vacuoles/lysosomes for recycling (Parzych and Klionsky, 2014; Delorme-Axford et al., 2015). Autophagy in filamentous fungi can be triggered by...
GFP-Atg8 to vacuoles was the result of autophagy within biofilm predominantly within vacuoles indicating autophagy had been activated. After 3 d of biofilm development, GFP-Atg8 signal appeared et al. could play roles in nutrient recycling in filamentous fungi (Shoji 2002). It has been suggested such pleomorphic vacuolar structures as previously reported for filamentous fungal vacuoles (Hyde 2013; Figure 6A, Post N starvation). The processing and accumulation of GFP-Atg8 gets delivered into vacuoles and, because GFP is resistant to proteolysis, the GFP signal accumulates within vacuoles (Pinar et al., 2013; Figure 6A, Post N starvation). The processing and accumulation of GFP-Atg8 within vacuoles after nitrogen starvation depend on other autophagy genes, including atg1 (Pinar et al., 2013). Therefore, in Atg1-deleted cells, GFP-Atg8 remains at PAS foci after nitrogen starvation (Figure 6B, atg1∆) as autophagy cannot occur.

Some vacuoles in our study were extended and tubular in shape, and can also occur in older basal cells of hyphae within colonies (Pollack et al., 2009; Voigt and Poggeler, 2013). We tracked GFP-Atg8, the A. nidulans orthologue of the yeast autophagy marker Atg8p (Klionsky et al., 2003; Xie et al., 2008), under conditions previously used to study nitrogen starvation-induced autophagy in A. nidulans (Pinar et al., 2013). We recapitulated the studies of Pinar et al., 2013, who defined GFP-Atg8 locations after nitrogen starvation. Prior to nitrogen limitation, GFP-Atg8 localizes throughout the cytoplasm as well as distinct foci (Figure 6A, Pre N starvation) that represent phagophore assembly sites (PAS) as previously reported (Pinar et al., 2013). After nitrogen starvation, to trigger autophagy, GFP-Atg8 gets delivered into vacuoles and, because GFP is resistant to proteolysis, the GFP signal accumulates within vacuoles (Pinar et al., 2013; Figure 6A, Post N starvation). The processing and accumulation of GFP-Atg8 within vacuoles after nitrogen starvation depend on other autophagy genes, including atg1 (Pinar et al., 2013). Therefore, in Atg1-deleted cells, GFP-Atg8 remains at PAS foci after nitrogen starvation (Figure 6B, atg1∆) as autophagy cannot occur.

Some vacuoles in our study were extended and tubular in shape, as previously reported for filamentous fungal vacuoles (Hyde et al., 2002). It has been suggested such pleomorphic vacuolar structures could play roles in nutrient recycling in filamentous fungi (Shoji et al., 2006a).

We next investigated if autophagy occurs during biofilm formation. After 3 d of biofilm development, GFP-Atg8 signal appeared predominantly within vacuoles indicating autophagy had been activated (Figure 6C, GFP-Atg8, Day 3). We confirmed this relocation of GFP-Atg8 to vacuoles was the result of autophagy within biofilm cells using a strain lacking Atg1 function (atg1∆). The GFP-Atg8 signal remained at PAS foci in biofilm cells lacking Atg1 (Figure 6D, GFP-Atg8 atg1∆). The data reveal that Atg1-dependent autophagy can be activated during biofilm maturation without media exchange to impose nitrogen starvation.

**Autophagy is required for biofilm maturation**

To determine if autophagy might play a role during biofilm formation, we asked if normal biofilm maturation was dependent on autophagy. Mature biofilms were allowed to develop over 7 d in strains with and without atg1 function and biofilm formation was measured using their dry weight. In the absence of autophagy caused by deletion of atg1, less than half the biomass of WT biofilms was able to be generated, indicating that autophagy is required for normal biofilm maturation (Figure 6E).

**Autophagy is activated by spinning disk confocal imaging of biofilm cells**

GFP-Atg8 locates to PAS foci and a general cytoplasmic signal in prebiofilm cells (Figures 6, A and C, and 7A, Growing cells, 0 Min image). This pattern does not change noticeably during live cell imaging at 20-s intervals for an hour (Figure 7A, Growing cells, 30 and 60 min). PAS foci maintained their overall intensity throughout the imaging period, as can be seen from the kymograph of a representative cell (Figure 7B). The overall cellular intensity of the GFP-Atg8 signal was additionally seen to slowly decrease slightly, presumably due to photo bleaching caused by the repeated imaging (Figure 7A, Growing cells graph).

Imaging of biofilm cells under identical conditions (at 20-s intervals for an hour) caused quite dramatic effects not observed in growing cells prior to biofilm formation. In biofilm cells, because autophagy had been triggered, GFP-Atg8 signal localized to vacuoles (Figure 7A, Biofilm cells, 0 Min). The GFP-Atg8 signal was not stable during imaging with the level of GFP fluorescence diminishing rapidly to below 10% the original levels and remained lowered for the rest of the imaging period (Figure 7A, Biofilm cells, 30 and 60 min, Biofilm cells graph). Although the overall level of GFP fluorescence decreased, an increase in the number of PAS foci was also surprisingly observed during imaging (Supplemental Video S1). These new PAS foci were transient in nature, forming then diminishing during imaging. When autophagy is triggered by nitrogen starvation, some PAS GFP-Atg8 foci mature into cup-shaped phagophores which develop into circular autophagosomes and then dissipate (Pinar et al., 2013). Identical PAS maturation events were observed during imaging of biofilm cells (Figure 8A and Supplemental Video S1). The GFP-Atg8 PAS maturation process triggered after nitrogen starvation can be seen as characteristic cone-shaped traces in kymographs (Pinar et al., 2013) which were also observed during imaging of biofilm cells (Figure 7C). In the particular biofilm cell shown in the Figure 7C kymograph, five sequential cone-shaped trace GFP-Atg8 PAS maturation events are apparent during the 1-h imaging period. The data indicate that autophagy can be triggered in biofilm cells during imaging. Prior imaging of autophagy tracking maturation of GFP-Atg8 PAS to phagophores after nitrogen starvation was carried out using 5-s delay image capture (Pinar et al., 2013). We therefore compared the response of GFP-Atg8 in biofilm cells when imaged either at 20-s intervals, as done in Figure 7, or at the shorter 5-s intervals. Notably, the response was similar under both protocols, although the photobleaching occurred more rapidly with the 5-s delays, as might be expected, and GFP-Atg8 PAS were still seen to mature into phagophores (Supplemental Figure S2).
We next investigated if the autophagy occurring during imaging of biofilm cells was triggered by the imaging light, or perhaps was something happening in cells throughout the imaging dish. We reasoned that if autophagy was triggered in response to imaging light it should not occur in adjacent unimaged cells. To investigate this, we repositioned the imaging dish after 1 h of imaging such that half the cells in the new field of view would be those previously imaged and half would not. In the previously imaged biofilm cells, GFP-Atg8 is located to PAS foci and phagophores, indicating autophagy had been triggered in the imaged cells. However, in the adjacent unimaged cells, all around those imaged, the GFP signal remains within vacuoles, indicating additional autophagy had not been triggered in them (Figure 8B). These observations indicate that, in biofilm cells expressing GFP-Atg8, autophagy is activated in response to the spinning disk confocal imaging light. To confirm the signals detected during light-activated autophagy in biofilm cells were dependent on GFP-Atg8, biofilm cells lacking any tagged protein were imaged in an identical manner. No signal was detected (Supplemental Figure S3). Finally, we tested if light-activated biofilm autophagy was dependent on Atg1 using a strain with atg1 deleted and did not observe any GFP-Atg8 modifications past PAS foci (Supplemental Figure S2, A, D, and E).

**DISCUSSION**

Our findings show that during fungal biofilm formation, the ER remains largely unchanged when tracking Sec63-GFP, an integral membrane ER protein. Sec63 is a component of the ER translocon complexes that mediate the delivery of proteins synthesized in the cytosol into the ER lumen. The ER structure is sensitive to stress and Sec63-GFP in A. nidulans collapses and aggregates during artificially induced ER stress (Markina-Inarrairaegui et al., 2013). The integrity of the ER within biofilm cells therefore indicates there is likely no general ER stress occurring within biofilm cells.

**Subcellular modifications during biofilm development**

While the ER appears apparently unmodified during biofilm maturation, subcellular changes do occur beyond the previously defined MT disassembly (Shukla et al., 2017), including modification of ERES, the Golgi, as well as the sites of endocytosis. Using fluorescently tagged marker proteins that locate to these structures, each was found to be dramatically modified in the


Autophagy during biofilm development

It is typical to starve cells and/or treat them with rapamycin to initiate autophagy, as previously done in studies of autophagy in *A. nidulans* (Pinar et al., 2013). However, our current study shows *A. nidulans* cells can trigger autophagy as biofilms form under growth conditions used to study fungal autophagy. Biofilm autophagy is dependent on the Atg1 protein kinase, indicating it represents activation of a normal autophagy pathway(s). We show autophagy is required for normal biofilm maturation as there is a reduction in biofilm biomass accumulation when atg1 is deleted, revealing there is a functional role for activation of autophagy during biofilm maturation. In another Aspergillus species, *Aspergillus oryzae*, deletion of atg1 prevents autophagy and causes defects in asexual development, conidiation, and sclerotial formation (Yanagisawa et al., 2013). It has also been shown in *A. oryzae* that autophagy is triggered in older basal cells of colonies which degrade peroxisomes, mitochondria, and nuclei within their vacuoles (Shoji et al., 2010). This study also revealed a role for autophagy in colony growth and expansion under nutrient-limited growth conditions (Shoji et al., 2006b) a similar situation also

![Figure 7: Autophagy is activated during spinning disk confocal imaging of biofilm founder cells expressing GFP-Atg8. (A) Plotting the levels of GFP fluorescence in growing nonbiofilm cells from GFP-Atg8 (strain MAD3474) during imaging at 20-s intervals over 1 h (blue line) and in similarly imaged biofilm cells (orange line). Error bars are SEM and n = 3. Inserted are images of representative cells at the start, middle, and end of imaging as indicated. (B) Kymograph showing GFP-Atg8 at a PAS in a growing nonbiofilm cell imaged at 20-s intervals for 1 h. (C) Kymograph showing GFP-Atg8 in a biofilm cell imaged at 20-s intervals for 1 h. Five examples of PAS formation and maturation into phagophores then autophagosomes, which dissipate, are numbered which display characteristic cone-shaped traces in kymographs during autophagy (Pinar et al., 2013). Bar, 5 μm.]
Autophagy has been shown to be activated within filamentous fungi and is self-generated hypoxia that promotes biofilm autophagy. In addition, reactive oxygen species (ROS) could be involved.

**Biofilm autophagy is reactivated by light**

Perhaps the most unexpected finding of this study is that spinning disk confocal light appears to activate autophagy in founder biofilm cells after they have already undergone autophagy. This is an intriguing finding. There is considerable interest in the photobiology of fungi (Idnurm et al., 2010; Idnurm, 2013; Corrochano, 2019) which perceive and respond to light to regulate many cellular processes including circadian rhythms, developmental processes, and stress responses (Corrochano, 2019). Several types of light receptor proteins have been identified in fungi including opsins, phytochromes, and cryptochromes (Yu and Fischer, 2019) and it remains to be determined if such proteins play a role in light-activated biofilm autophagy. However, another potential photoreceptor in our experiments, GFP, might also play a role.

Fluorescent proteins, including GFP species, have the capacity to generate ROS when illuminated and can act as genetically encoded photosensitizers to produce singlet oxygen and superoxide on illumination (Trewin et al., 2018). This feature is utilized in chromophore-assisted laser inactivation (Jacobson et al., 2008; Jarvela and Linstedt, 2014; Sano et al., 2014; Wojtovich and Foster, 2014) and has led to the design of fluorescent proteins with higher yield of ROS such as KillerRed (Bulina et al., 2006) and SuperNova (Takemoto et al., 2013). It is therefore possible that GFP in our experiments produces ROS during imaging illumination, particularly within biofilm cells. This would be consistent with the rapid reduction in GFP fluorescence during imaging of biofilm cells being the result of ROS-mediated photo bleaching (Greenbaum et al., 2000). Notably, the GFP signal within growing nonbiofilm cells is not sensitive to bleaching during imaging. ROS has previously been implicated as a trigger for autophagy (Azad et al., 2009; Scherz-Shouval and Elazar, 2011) with ROS potentially acting as signaling molecules (Scherz-Shouval et al., 2007). Alternatively, ROS-mediated protein and lipid damage might generate suitable substrate targets for autophagy leading to its activation.

There is precedent for light-activated ROS via GFP-like proteins being able to trigger autophagy. For example, targeting light-activated ROS generators to mitochondria promotes activation of mitophagy (Yang and Yang, 2011; Wang et al., 2012). Also, observed in Aspergillus fumigatus (Richie and Askew, 2008). Autophagy within the Aspergilli therefore seems to play roles during differentiation, colony growth and biofilm formation with these roles being more prominent in nutrient-poor conditions, including limited metal ions (Richie et al., 2007; Richie and Askew, 2008).

Further studies will be required to see what autophagy activation pathway(s) lead to biofilm-regulated autophagy (Corona Velazquez and Jackson, 2018), beyond the involvement of Atg1, and to better understand what mode(s) of autophagy are activated (Galluzzi et al., 2017). Given that hypoxia is generated within fungal biofilms and that hypoxia is a known trigger for autophagy (Mazure and Pouyssegur, 2010; Blagosklonny, 2013; Tan et al., 2016), it is possible that it is self-generated hypoxia that promotes biofilm autophagy.

Nutrient resources recycled within the older founder biofilm cells via autophagy could be made available to support growth or other processes, such as extracellular matrix formation, toward the biofilm surface. Based on proteomics, it has been suggested that selective ribosome autophagy might occur in Candida orthopsilosis biofilms (Pires et al., 2016), but our study might be the first to directly demonstrate activation of autophagy within a fungal biofilm. In addition, autophagy has been shown to be activated within filamentous fungal plant pathogens as they encounter nutrient-poor conditions during the infection process (Kershaw and Talbot, 2009; Zhu et al., 2019) and autophagy is required for fungal pathogenesis (Zhu et al., 2019). It will be interesting to investigate what microenvironmental changes might be responsible for activation of biofilm autophagy (Corona Velazquez and Jackson, 2018) but, as discussed further below, it is possible that reactive oxygen species (ROS) could be involved.
nonmitochondrial-generated ROS can lead to activation of autophagy (Hoffmann et al., 2019) and ROS generated in lysosomes promotes their autophagic turnover in a process termed lysophagy (Hung et al., 2013).

Another mechanism by which light might cause reactivation of autophagy is through effects on the cellular energetic balance within founder biofilm cells. After biofilm autophagy has occurred, cells might reach a metabolic state incompatible with further autophagy. For example, because autophagy is an energy-dependent process, a limited capacity to generate sufficient ATP could hinder autophagy (Schellenis et al., 1988; Singh and Cuervo, 2011). If this was the case, the mechanism by which light reactivates autophagy could be by promoting increased ATP levels. This could involve NO, which is known to inhibit cytochrome c oxidase in a manner that is reversible by light (Lane, 2006; Hamblin, 2018; Osipov et al., 2018).

CONCLUSIONS

Not many studies of fungal biofilm biology have addressed what happens to cellular architecture as biofilms develop. Our study reveals that as biofilms form and mature, the founder cells modify their cytoskeleton and the apparatus for exocytosis and endocytosis in a manner that requires the hypoxic transcription factor SrbA. Many genes have been shown to be regulated by SrbA to enable cells to survive hypoxia and our results suggest part of the SrbA-regulated response is to enable cells to enter a more dormant-like state. We speculate that part of the reason cells lacking SrbA cannot survive hypoxia is their inability to down-regulate the cellular processes needed to enter a less active state. Autophagy is also shown to be a modifier of cellular structure within biofilm cells and we find imaging light is able to trigger biofilm autophagy, potentially via illuminated GFP generation of ROS. There is great interest in light therapies and their mode of action (Serrage et al., 2019) and our study, in a more general sense, provides additional evidence suggesting that light-activated autophagy (Kessel and Oleinick, 2009; Kessel et al., 2013) should be considered as a potential contributing factor in such treatment modalities.

MATERIALS AND METHODS

Request a protocol through Bio-protocol.

General techniques

Classical genetics, strain construction, media preparation, culture, and transformation of A. nidulans were carried out as described previously (Pontecorvo et al., 1953; Yang et al., 2004; Nayak et al., 2006; Shukla et al., 2017). Strains used in the study, genotypes, and source are listed in Table 1.
Biofilm formation and microscopy
Biofilm cultures were generated and tracked microscopically as described previously (Shukla et al., 2017). For autophagy experiments, the nitrogen source and concentration were as used previously to study autophagy in A. nidulans (Pinar et al., 2013). We did not add rapamycin during nitrogen starvation. For biofilm formation, conidia were inoculated at 2.5 × 10^5/ml in 3 ml minimal media in glass coverslip–bottomed dishes (MatTek, Ashland, MA), grown at room temperature over a 3-d period, and imaged each day such that prebiofilm and maturing biofilm cells were imaged. To measure biofilm maturation, biomass accumulated over 7 d of growth was determined as the dry weight of cells after desiccating for at least 3 d in a 50°C drying oven after media removal by blotting between paper towels. Spinning disk confocal systems (UltraVIEW ERS and UltraVIEW Vox CSUX1; PerkinElmer) were used as described previously (Shukla et al., 2017; Suresh et al., 2017). Images are presented as maximum intensity projections except those in Figure 1, which are single z images. ImageJ (National Institutes of Health) software was used to quantify GFP levels by using Threshold settings to limit measured pixels to within cells, and measurements were taken using the Analyze Particles command. Values were then normalized as a percentage of total signal present in the first imaged cells.

NO treatment
NOC-12 was used from a stock 100 mM solution, made in 10 mM NaOH, to yield final concentrations between 50 and 100 µM to generate NO. The addition of the maximum levels of 10 mM NaOH used (30 µl in 3 ml) did not affect the pH of the buffered media and did not affect any of the proteins monitored. For the NO experiments, early stage prebiofilm cultures were used with NOC-12 being added and mixed into the culture media prior to imaging.

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