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REVIEW

Molecular Mechanisms of Conidial Germination in *Aspergillus* spp.

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SUMMARY Aspergilli produce conidia for reproduction or to survive hostile conditions, and they are highly effective in the distribution of conidia through the environment. In immunocompromised individuals, inhaled conidia can germinate inside the respiratory tract, which may result in invasive pulmonary aspergillosis. The management of invasive aspergillosis has become more complex, with new risk groups being identified and the emergence of antifungal resistance. Patient survival is threatened by these developments, stressing the need for alternative therapeutic strategies. As germination is crucial for infection, prevention of this process might be a feasible approach. A broader understanding of conidial germination is important to identify novel antigermination targets. In this review, we describe conidial resistance against various stresses, transition from dormant conidia to hyphal growth,

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the underlying molecular mechanisms involved in germination of the most common *Aspergillus* species, and promising antigermination targets. Germination of *Aspergillus* is characterized by three morphotypes: dormancy, isotropic growth, and polarized growth. Intra- and extracellular proteins play an important role in the protection against unfavorable environmental conditions. Isotropically expanding conidia remodel the cell wall, and biosynthetic machineries are needed for cellular growth. These biosynthetic machineries are also important during polarized growth, together with tip formation and the cell cycle machinery. Genes involved in isotropic and polarized growth could be effective antigermination targets. Transcriptomic and proteomic studies on specific *Aspergillus* morphotypes will improve our understanding of the germination process and allow discovery of novel antigermination targets and biomarkers for early diagnosis and therapy.

KEYWORDS *Aspergillus*, conidia, dormant, germination, isotropic growth, polarized growth

INTRODUCTION

For reproduction or to survive hostile conditions, aspergilli produce asexual conidia on specialized hyphal structures, called conidiophores, that extend up to 100 μm into the air. This height is suggested to be high enough for outdoor turbulent airflow to transport conidia into the air (1). The distribution of *Aspergillus* conidia through the environment is highly efficient (2), demonstrated by the observation that *Aspergillus* was the most common fungal genus in indoor air sampling (3). Environmental surveys indicate that humans inhale 100 to 1,000 *Aspergillus fumigatus* conidia each day, and because of their small size (2 to 3 μm), some will reach the lung alveoli (4). Under specific conditions the fungus may cause disease in humans, and *A. fumigatus* is the most frequent cause of *Aspergillus* diseases in the majority of regions in the world (5).

Outgrowth of *Aspergillus* conidia inside human lungs is the first step of infection in patients who have a weakened immune system or chronic lung disease. *Aspergillus* species cause a spectrum of different diseases, of which the most serious form is invasive aspergillosis (6). Mortality as a result of invasive aspergillosis is significant and could potentially increase to 90% in specific patient groups (7). Various non-*fumigatus* *Aspergillus* species may have different clinical presentations or involve diverse patient groups (8). Compared with those with *A. fumigatus* infection, patients with invasive aspergillosis due to non-*fumigatus* *Aspergillus* species, e.g., *A. flavus*, *A. niger*, and *A. terreus*, more commonly developed disseminated infection and paranasal sinus involvement (9).

The management of invasive aspergillosis has become more complex, with new risk groups being identified, such as patients treated with ibrutinib and invasive aspergillosis associated with influenza (10, 11), and the emergence of antifungal drug resistance (12). *Aspergillus* species may be intrinsically resistant to antifungal drug classes, such as the polyenes in the case of *A. terreus* and the azoles in the case of *A. calidoustus* (13, 14). Furthermore, taxonomic changes have identified numerous new cryptic species with diverse resistance profiles (15). A prevalence of 7.2% cryptic species was found in a recent study from Portugal, with azole resistance percentages ranging from 47% for voriconazole to 100% for isavuconazole (16). In addition, acquired resistance is an emerging problem in *Aspergillus* species, especially against the azole class (12). Azole resistance is most commonly reported for *A. fumigatus* but is also observed in other species (17). In *A. fumigatus*, environmental exposure of the fungus to azole fungicides was found to be a major driver of resistance selection (18). Furthermore, voriconazole resistance was found to reduce patient survival by 20% to 30% compared with voriconazole-susceptible invasive aspergillosis (19). As only three drug classes are available for treatment of *Aspergillus* diseases, with the azole class being most prominently used, alternative treatment options are very limited in cases of drug-resistant aspergillosis.

These developments threaten patient survival, and new treatment strategies are

needed to improve our ability to manage this infection. Prevention of *Aspergillus* diseases has been shown to be a feasible approach in invasive aspergillosis but involves azole drugs, as these are the only oral treatment option. In the setting of acquired environmental resistance, azole prophylaxis might select for resistant infection rather than preventing it, underscoring the need to explore alternative preventive strategies. As prevention of inhalation of *Aspergillus* conidia is unrealistic, interventions that interact with germination of conidia in the lung might be a feasible approach. Therefore, a broader understanding of the germination process in *A. fumigatus* may help to identify novel antigermination targets.

Since the genome sequences for eight aspergilli have become available (20–26), transcriptomic studies focusing on the molecular mechanisms underlying spore germination have further increased, on pathogenic aspergilli as well as on nonpathogenic species (27–34). More recently, 10 novel genome sequences of the *Aspergillus* genus became available (35), allowing more comparative analyses within the fungal genus. The studies at the mRNA level help to reveal mechanisms of the transition from dormant conidia to germinating conidia and could provide ample information for possible therapeutic targets. However, these transcriptomic studies alone are not sufficient and should be extended by relevant studies at the protein level. For pathogenic aspergilli, various proteomic studies have been carried out to understand the germination process (36–42). This review is focused on the molecular mechanisms involved in conidial germination of *A. fumigatus*, *A. niger*, *A. nidulans*, *A. flavus*, and *A. terreus*, from dormant conidia to germ tube formation. Comparative analysis of dormant conidia and the transition to hyphal growth on different aspergilli may help to determine common active processes during germination. This may lead to the identification of specific or common therapeutic targets and biomarkers for early diagnostics and therapy.

PHASES IN THE GERMINATION OF CONIDIA

Dormant conidia are stress-tolerant microscopic structures, and due to their intra- and extracellular characteristics, they are able to survive unfavorable conditions such as thermal stress, dehydration, osmotic pressure, oxidative stress, variations in pH, and UV. Figure 1 shows a schematic representation of the conidial cell. Dormant conidia are able to germinate even after 1 year of storage at room temperature (33). The presence of nutrients such as inorganic salts, sugars, and amino acids is required for conidial germination in most filamentous fungi (43). In *A. fumigatus*, dormancy is broken upon exposure to a degradable carbon source in the presence of water and air (44), whereas the presence of glucose alone is enough to break dormancy in *A. nidulans* (45). Activation of dormant conidia leads to an increased intracellular osmotic pressure, followed by water uptake (44, 46). During this process the conidia start to swell and the cellular diameter increases 2-fold (29, 30). The swelling phase of conidia is also called isotropic growth. Swollen conidia direct the growth to one side of the cell to grow in a polarized fashion, which leads to the formation of a germ tube. The phase resulting in the formation of a germ tube is also called polarized growth. Figure 2 shows the morphology of the different growth phases during germination; underlying biological processes for each phase are indicated in boxes. In the next sections, the focus is aimed at dormant conidia and their stress resistance, breaking of dormancy, regulatory pathways involved in germination, and the transcriptomic and proteomic landscape during the isotropic and polarized growth phases.

DORMANT CONIDIA

An overview of gene transcripts and proteins found in dormant conidia is provided in Table 1. Transcriptomic and proteomic studies reported many transcripts and proteins present in dormant conidia, but in this review, only transcripts and proteins important for conidial survival and viability are included.

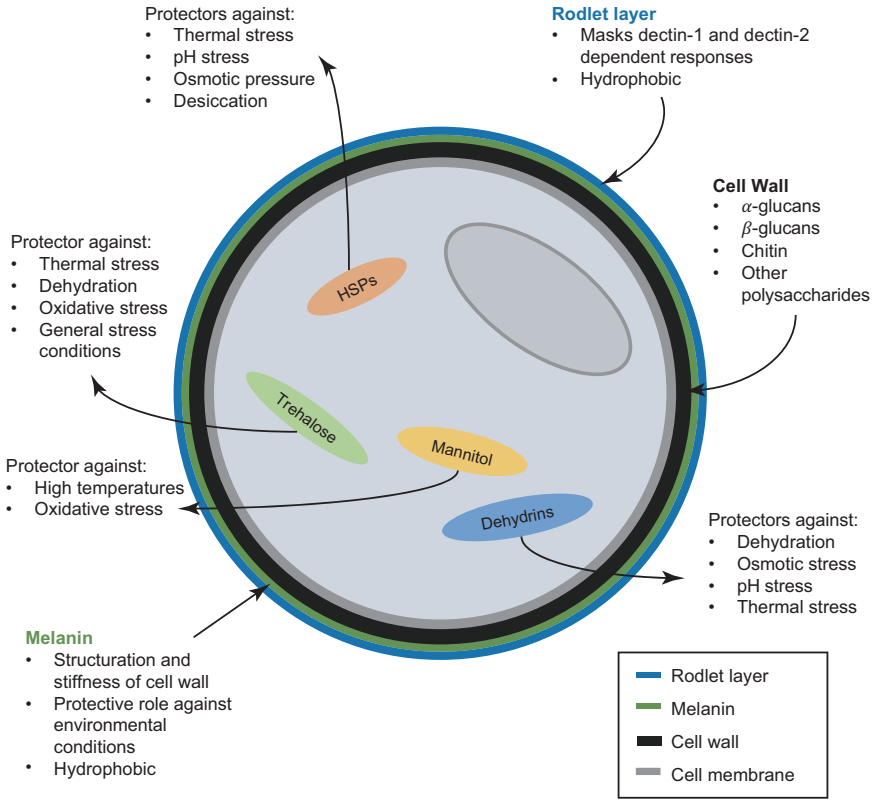

FIG 1 Schematic representation of the conidial cell.

Central Regulators of Conidiation

After a period of mycelial growth, conidia are formed on specialized aerial hyphae, called conidiophores. Conidiophore and conidium formation in *Aspergillus* is highly conserved and is controlled by the central regulatory proteins BrlA, AbaA, and WetA (Fig. 3) (47, 48). Activation of *brlA* is essential for conidiophore development in *A. fumigatus* and *A. nidulans* (49, 50). BrlA is a C_2H_2 zinc finger transcription factor (49) and controls other conidiation genes, including *abaA*, *wetA*, *rodA*, and *yA* (51). These genes have multiple BrlA binding sites or BrlA response elements (BREs) in their promoter regions; additionally, in *A. fumigatus*, BREs are present in *abaA*, *wetA*, *rodA*, and the *velvet* regulators *vosA* and *velC* (Fig. 3) (52). A previous study has identified the response elements for *A. nidulans* BrlA, which are 5′-(C/A)(G/A)AGG(G/A)-3′) (53). *brlA* is expressed during the early phase of conidiation. In the absence of *brlA*, expression of *abaA*, *wetA*, *vosA*, and *rodA* was eliminated, indicating that *brlA* acts upstream of *abaA*, *wetA*, *vosA*, and *rodA* (52). *abaA* accumulates during the middle to late phase of conidiation and is activated by BrlA (52, 54). In *A. fumigatus*, AbaA binding sites or AbaA response elements (AREs) are present in the promoter regions of *brlA*, *wetA*, *rodA*, *vosA*, *veA*, *velB*, *velC*, and *abaA* itself (Fig. 3) (52). Another study identified the response elements for *A. nidulans* AbaA, which are 5′-CATTCY-3′, where Y is a pyrimidine (55). AbaA activates *wetA* during the late phase of conidiation.

WetA is essential for the completion of conidial maturation; in a *wetA*-defective mutant, normal conidiophores are formed. However, conidia are white, take up water, and autolyze instead of going into the final stages of maturation (52, 56, 57). Δ*wetA* conidia lack pigment and are defective in synthesizing the conidial cell wall (52, 56–58). Moreover, transcripts of genes associated with the biosynthesis of trehalose, hydrophobins, and the degradation of β-glucan decreased in Δ*wetA* conidia (trehalose, hydrophobins, and the cell wall are discussed below). Vice versa, transcripts of genes associated with biosynthesis of chitin, and β-(1,3)-glucans increased in Δ*wetA* conidia (57). Δ*wetA* conidia lacked internal trehalose; these

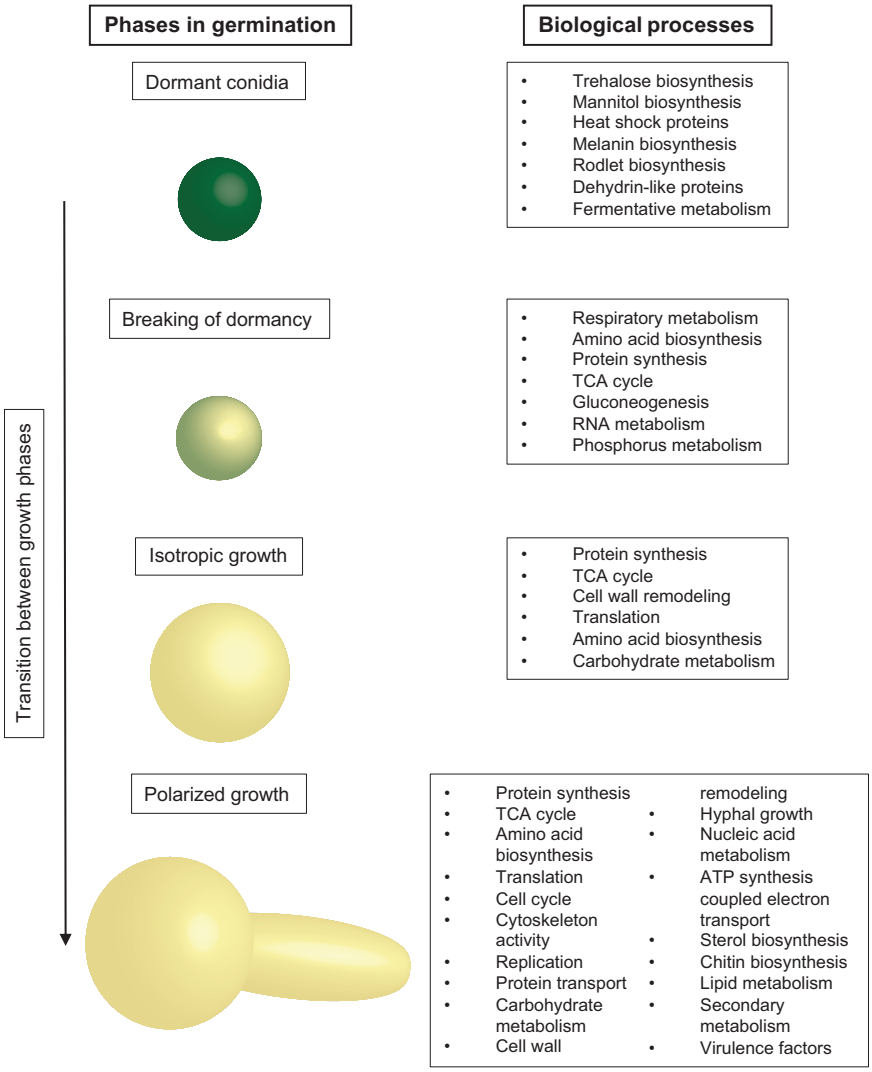

FIG 2 Transition between different morphotypes during germination. Important biological processes for each morphotype are mentioned in boxes.

conidia exhibited greatly reduced tolerance to osmotic, oxidative, and thermal stresses. Transmission electron microscopy (TEM) analysis of Δ*wetA* mutants indicated that the majority of conidia were misshaped and lacked cytoplasm. A recent study identified putative WetA response elements (WREs), which are 5′-CCGYTTGC GGC-3′ (Fig. 3) (59). WREs were found in the *velvet* regulators *vosA* and *velB* and in *wetA* itself. The *wetA* DNA-binding motif was found in *A. nidulans*, *A. fumigatus*, and *A. flavus*. WetA and the other two central regulators were present in *Aspergillus*, *Penicillium*, and *Talaromyces* species (60). WetA in *Aspergillus* is functionally conserved and plays an important role in conidiation as well as in many aspects of conidial biology, including cell wall integrity (CWI), stress tolerance, and spore viability. *A. fumigatus* WetA functionally diverges in its regulation of the melanin biosynthesis pathway (59). AbaA, BrlA, and WetA are defined as the central regulatory pathway for conidium formation and maturation (BrlA→AbaA→WetA). This central regulatory pathway is reviewed in more detail by Adams et al. (51), Yu (48), and Park and Yu (61).

Regulatory Proteins

The *velvet* family of regulatory proteins. The *velvet* regulators share the *velvet* DNA-binding motif and play a key role in coordinating fungal growth, differentiation, and

TABLE 1 List of proteins and genes expressed in dormant conidia

Gene or protein	*Aspergillus* species	Systemic name	Description	Reference(s)
Gene				
tpsC	*A. niger*	An14g02180	Trehalose biosynthesis gene. Trehalose is important for conidial survival.	30
tppB	*A. niger*	An13g00400	Trehalose biosynthesis gene. Trehalose is important for conidial survival.	30
tppC	*A. niger*	An07g08720	Trehalose biosynthesis gene. Trehalose is important for conidial survival.	30
tpsA	*A. fumigatus*	Afu6g12950	Trehalose biosynthesis gene. Trehalose is important for conidial survival.	81
	A. niger	An08g10510	Trehalose biosynthesis gene. Trehalose is important for conidial survival.	30
tpsB	*A. fumigatus*	Afu2g04010	Trehalose biosynthesis gene. Trehalose is important for conidial survival.	81
mtdA	*A. niger*	An15g05450	Gene for putative mannitol dehydrogenase; transcripts found abundantly present in dormant conidia. Mannitol is important for conidial survival.	30
mpdA	*A. niger*	An02g05830	Gene for putative mannitol dehydrogenase; transcripts found abundantly present in dormant conidia. Mannitol is important for conidial survival.	30
hsp90	*A. fumigatus*	Afu5g04170	Gene for heat shock protein important for spore viability, germination, hyphal growth, and conidiation.	90
wetA	*A. fumigatus*	Afu4g13230	Essential for trehalose biogenesis in conidia; gene for developmental regulatory protein involved in conidial development.	52
	A. niger	An06g01610	The gene encodes a protein homologous to HSP9 of *S. pombe* and HSP12 of *S. cerevisiae*. In *S. cerevisiae*, it protects the plasma membrane against desiccation.	30
	A. niger	An01g13350	The gene encodes a homologue of HSP104 of *S. cerevisiae*, which is of importance to thermotolerance, ethanol tolerance, and sodium arsenite tolerance.	30
	A. niger	An15g05410	Gene for putative heat shock protein; transcripts found in dormant conidia.	30
	A. niger	An07g09990	Gene for HSP70 protein; transcripts found in dormant conidia.	30
	A. niger	An18g00600	Gene for putative heat shock protein; transcripts found in dormant conidia.	30
arp1	*A. fumigatus*	Afu2g17580	Conidial DHN melanin biosynthesis gene.	29
arp2	*A. fumigatus*	Afu2g17560	Conidial DHN melanin biosynthesis gene.	29
ayg1	*A. fumigatus*	Afu2g17550	Conidial melanin biosynthesis gene.	29
mcoC	*A. niger*	An03g03750	Conidial melanin biosynthesis gene.	30, 102
fwnA	*A. niger*	An09g05730	Conidial melanin biosynthesis gene.	30, 102
ayg1	*A. niger*	An14g05350	Conidial melanin biosynthesis gene; mutants produce gray conidia and small conidiophores.	30, 102
dprA	*A. fumigatus*	Afu4g00860	Encodes a dehydrin-like protein, which plays a role in the oxidative stress response.	72, 74
dprB	*A. fumigatus*	Afu6g12180	Encodes a dehydrin-like protein, which plays a role in osmotic and pH stress responses.	74
dprC	*A. fumigatus*	afu7g04520	Encodes a dehydrin-like protein, which plays a role in tolerance against freezing.	109
Protein				
WetA	*A. fumigatus*	Afu4g13230	Regulatory protein involved in conidiation as well as cell wall integrity, stress tolerance, and spore viability.	52, 56–58
	A. nidulans	AN1937		
VosA	*A. fumigatus*	Afu4g10860	Regulatory protein involved in conidiation. The VelB-VosA heterodimer is required for spore maturation, trehalose biogenesis, long-term viability of conidia, and resistance to stresses and negatively controls spore germination.	63, 65–68
	A. nidulans	AN1959		
	A. flavus	AFL2G_01912		

(Continued on next page)

TABLE 1 (Continued)

Gene or protein	*Aspergillus* species	Systemic name	Description	Reference(s)
VelB	*A. fumigatus*	Afu1g01970	Regulatory protein involved in conidiation. The VelB-VosA heterodimer is required for spore maturation, trehalose biogenesis, long-term viability of conidia, and resistance to stresses and negatively controls spore germination.	63, 65–68
	A. nidulans	AN0363		
	A. flavus	AFL2G_00875		
VeA	*A. fumigatus*	Afu1g12490	Regulatory protein involved in conidiation. The VelB-VeA heterodimer negatively controls spore germination.	63, 65–68
	A. nidulans	AN1052		
	A. flavus	AFL2G_ 07468		
LaeA	*A. nidulans*	AN0807	Non-*velvet* regulatory protein. The formation of the VelB-VosA heterodimer complex is directed by LaeA.	63, 64
GanB	*A. nidulans*	AN1016	GanB plays a role in controlling conidial germination by mediating the cAMP/PKA signaling pathway.	120, 125
MybA	*A. fumigatus*	Afu3g07070	Involved in the control of conidial formation and maturation; plays an essential role in the survival of the conidia.	70
CspA	*A. fumigatus*	Afu3g08990	Repeat-rich GPI-anchored cell wall protein. CspA is involved in spore viability and cell wall permeability.	70
AtfA	*A. fumigatus*	Afu3g11330	Basic-region leucine zipper transcription factor. Plays a role in the response of conidia to stress.	32, 71–73
	A. nidulans	AN2911		
	A. niger	An02g07070		
	A. oryzae	AO090003000685		
TslA	*A. fumigatus*	Afu7g03940	Trehalose-related regulatory protein which interacts with chitin synthase CsmA. Δ*tslA* mutants have an altered cell wall structure.	83
CsmA/ChsE	*A. fumigatus*	Afu2g13440	Class V chitin synthase which interacts with trehalose-related regulatory protein TslA.	83
MpdA	*A. fumigatus*	Afu2g10660	Putative mannitol dehydrogenase found abundantly present in dormant conidia. Mannitol is important for conidial survival.	38, 39
Scf1	*A. fumigatus*	Afu1g17370	Putative heat shock protein enriched in dormant conidia.	38
Awh11	*A. fumigatus*	Afu6g12450	Putative heat shock protein enriched in dormant conidia.	38
BipA	*A. fumigatus*	Afu2g04620	HSP70 chaperone enriched in dormant conidia.	38
HSP70	*A. fumigatus*	Afu1g07440	Molecular chaperone enriched in dormant conidia.	38
ClxA	*A. fumigatus*	Afu4g12850	Putative calnexin enriched in dormant conidia.	38
Egd2	*A. fumigatus*	Afu6g03820	Predicted role in protein folding. Protein enriched in dormant conidia.	38
Hsp30	*A. fumigatus*	Afu3g14540	Putative heat shock protein enriched in dormant conidia.	37
RodA	*A. fumigatus*	Afu5g09580	Conidial hydrophobin required for formation of the rodlet layer of conidia.	36, 37, 95, 104–107
	A. nidulans	AN8803		
Abr2	*A. fumigatus*	Afu2g17530	Protein involved in conidial melanin biosynthesis.	37, 38
Arp1	*A. fumigatus*	Afu2g17580	Conidial DHN melanin biosynthesis protein.	37, 38
Pep2	*A. fumigatus*	Afu3g11400	Protein enriched in conidia; cell surface associated.	36
	A. fumigatus	Afu5g02040	Putative extracellular lipase enriched in conidia.	36
PdiA	*A. fumigatus*	Afu2g06150	Protein enriched in conidia; cell surface associated.	36
	A. fumigatus	Afu7g06750	Protein enriched in conidia; cell surface associated.	36
Aspf3	*A. fumigatus*	Afu6g02280	Allergen Asp f3; cell surface associated.	36
CipC	*A. fumigatus*	Afu5g09330	Protein enriched in conidia; cell surface associated.	36
	A. fumigatus	Afu7g04210	Protein enriched in conidia; cell surface associated.	36
	A. fumigatus	Afu2g11060	Protein enriched in conidia; cell surface associated.	36
Wos2	*A. fumigatus*	Afu5g13920	Putative HSP90 binding cochaperone; cell surface associated.	36
Rpp1	*A. fumigatus*	Afu1g06830	Putative 60S acidic ribosomal protein super family member; cell surface associated.	36
CcpA	*A. fumigatus*	Afu1g13670	Conidial surface protein highly abundant in resting conidia; CcpA reduces recognition by the innate immune system.	107

(Continued on next page)

TABLE 1 (Continued)

Gene or protein	*Aspergillus* species	Systemic name	Description	Reference(s)
DlpA	*A. nidulans*	AN5324	Dehydrin-like protein which plays a role osmotic and thermal stresses.	110
CatA	*A. fumigatus*	Afu6g03890	Conidium-specific catalase CatA.	72, 75
Scf1	*A. fumigatus*	Afu1g17370	Putative heat shock protein enriched in dormant conidia.	72
ConJ	*A. fumigatus*	Afu6g03210	Putative conidiation gene; deletion of *conF* and *conJ* results in delayed germination and decreased desiccation resistance.	72, 76
	A. nidulans	AN5015		
MpkC	*A. fumigatus*	Afu5g09100	Putative MAPK involved in the oxidative stress response; transcript abundance increases in response to carbon source and oxidative stress.	72, 113–116
	A. nidulans	AN4668		
Pbs2	*A. fumigatus*	Afu1g15950	Mitogen MAPKK of the HOG signaling pathway that regulates osmotic stress response.	72, 113, 114
PbsA	*A. nidulans*	AN0931		
SakA	*A. fumigatus*	Afu1g12940	Putative MAPK with predicted roles in the osmotic and oxidative stress responses.	72, 113–116
HogA/SakA	*A. nidulans*	AN1017		
OsrA	*A. fumigatus*	NA[a]	Hypothetical osmotic stress regulator.	74
SskB	*A. nidulans*	AN10153	MAPKKK of the HOG signaling pathway that regulates the osmotic stress response.	113
SskA	*A. nidulans*	AN7697	Response regulator; part of a two-component signal transducer involved in the HOG signaling pathway that regulates the osmotic stress response.	113
TcsB	*A. nidulans*	AN1800	Transmembrane histidine kinase; part of a two-component signal transducer involved in the HOG signaling pathway that regulates the osmotic stress response.	113
YpdA	*A. nidulans*	AN2005	Histidine-containing phosphotransfer protein; part of a two-component signal transducer involved in the HOG signaling pathway that regulates the osmotic stress response.	113

[a]NA, nonapplicable.

secondary metabolism in ascomycetes and basidiomycetes (62). An evolutionary study showed the presence of the *velvet* regulators in 16 *Aspergillus* species (60). The *velvet* regulators include VeA, VelB, VelC, and VosA. Some *velvet* proteins form dynamic complexes with each other and with the non-*velvet* regulatory protein LaeA to regulate various processes in filamentous fungi (63–66). Importantly, these proteins are key regulators of conidiation; however, a role in maturation and viability of spores has also been described (Fig. 3) (67, 68). Genetic studies demonstrate that AbaA positively regulates *vosA* and *velB* expression during the middle and late phases of conidiation by binding directly to the promoters of the genes (Fig. 3) (65). The formation of nuclear VelB-VosA heterodimeric complex is directed by LaeA (64). The VelB-VosA heterodimer is required for spore maturation, trehalose biogenesis, long-term viability of conidia, and resistance to thermal, oxidative, and UV stresses (64, 67–69). The VelB-VosA heterodimer negatively controls germination, as Δ*velB* Δ*vosA* double mutants exhibited increased germination (65). Similarly, Δ*veA*, Δ*velB*, and Δ*veA* Δ*velB* mutants showed increased germination rates in *A. fumigatus* (67). It is proposed that VelB acts downstream of GanB (AN1016) in controlling conidial germination, as 60% of the Δ*velB* Δ*ganB* double mutants germinated after 4 h of incubation (65). GanB plays a role in controlling conidial germination by mediating the cyclic AMP (cAMP)-protein kinase A (PKA) signaling pathway. This pathway is discussed in more detail below.

MybA and AtfA. A novel transcription factor involved in conidiation and viability of the conidia was identified in *A. fumigatus* and designated MybA (70). MybA is involved in the regulation of asexual development; however, this novel regulator is not controlled by the central regulators BrlA and AbaA. MybA controlled the expression of *wetA* and *velvet* regulators *vosA* and *velB* in *A. fumigatus* (Fig. 3) (70). Possible binding sites

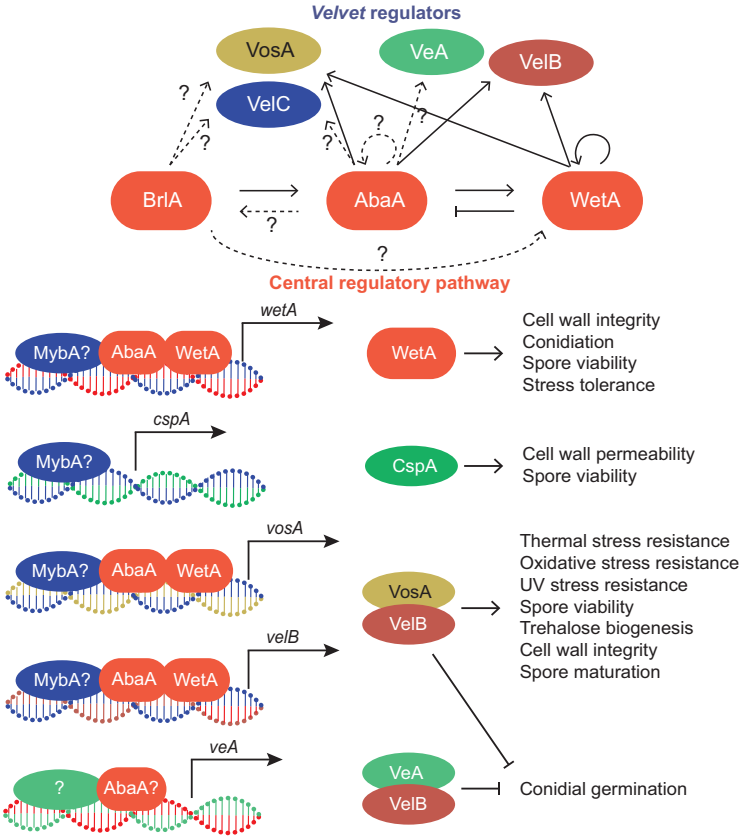

FIG 3 Regulatory relationship between the central regulators, *velvet* proteins, and MybA. BrlA, AbaA, and WetA are defined as the central regulators for conidiation. *abaA*, *wetA*, *velC*, and *vosA* have putative BrlA response elements [BREs; 5′-(C/A)(G/A)AGG(G/A)-3′] in their promoter regions. *brlA*, *wetA*, *velC*, *vosA*, *velB*, *veA*, and *abaA* itself have putative AbaA response elements (AREs; 5′-CATTCY-3′, where Y is a pyrimidine) in their promoter regions. *vosA*, *velB*, and *wetA* have putative WetA response elements (WREs; 5′-CCGY TTGCGGC-3′) in their promoter regions. WetA also plays a role in cell wall integrity, spore viability, and stress tolerance. AbaA positively regulates expression of *wetA*, *vosA*, and *velB*. These genes potentially have MybA binding sites in their promoters. Additionally, *cspA* expression was totally switched of in a Δ*mybA* mutant. MybA, so far, has been described only for *A. fumigatus*. CspA is involved in spore viability and cell wall permeability. The VelB-VosA heterodimer is involved in spore maturation, cell wall integrity, trehalose biogenesis, spore viability, and thermal, oxidative, and UV stress resistance. The VelB-VosA and VelB-VeA heterodimers negatively control germination.

for MybA were identified in the promoter regions of *wetA*, *vosA*, and *velB* (70). A transcriptome sequencing (RNA-Seq) experiment showed one gene totally switched of in a Δ*mybA* mutant, which was the repeat-rich glycosylphosphatidylinositol (GPI)-anchored cell wall protein CspA. Δ*mybA* and Δ*cspA* showed defects in conidial survival and cell wall permeability; in Δ*mybA* mutants upregulation of many cell wall biosynthesis genes was observed, including all α- and β-(1,3)-glucan synthase and several chitin synthase genes (70). MybA was also involved in trehalose biosynthesis, as the Δ*mybA* mutants showed reduction or complete loss of trehalose content. Loss of trehalose is associated with decreased viability (52, 65, 67). Additionally, in the Δ*mybA* mutants, genes involved in scavenging reactive oxygen species (ROS) were downregulated. ROS scavenging proteins are needed for protection against apoptotic death induced by stress.

An earlier-characterized bZip (basic-region leucine zipper)-type transcription factor, AtfA, was identified in several *Aspergillus* species with reference to germination and conidial stress tolerance (32, 71–73). AtfA positively regulates genes related to stress tolerance and negatively regulates genes related to germination (32). Δ*atfA* resting conidia started germinating without any available nutrients, suggesting that *atfA* plays a role in conidial dormancy. Additionally, early expression of germination-associated

gene *calA* and an uncommon metabolic activity were observed in Δ*atfA* conidia. The Δ*atfA* mutant was increasingly more sensitive to stresses such as oxidative stress and thermal stress (72). Transcriptomic analysis identified four stress protection-related genes regulated by AtfA at the conidiation stage (72). Those genes encoded the conidium-specific catalase CatA, the dehydrin-like protein DprA, putative heat shock protein (HSP) Scf1, and the conidiation-specific protein ConJ. DprA and CatA both were involved in resistance to oxidative stress (74, 75), HSPs play a role against various stresses, and ConJ, together with ConF, is involved in desiccation stress and conidial germination (76).

Compatible Solutes

Aspergillus spp. protect themselves against drought, heat, and other stressors by accumulating compatible solutes. Important solutes are sugars, sugar alcohols, amino acids, and betaine (77). One of those solutes is trehalose, a sugar consisting of two glucose units. Trehalose is essential for stress resistance, particularly against thermal stress and dehydration, and for long-term viability (Fig. 1) (69, 78). In *A. nidulans* and *A. niger*, trehalose accumulated in dormant conidia and was degraded during germination (79, 80). Transcripts of the trehalose biosynthesis genes *tpsA*, *tpsC*, *tppB*, and *tppC* were found in dormant conidia of *A. niger* (30). Transcript levels of these genes decreased strongly 2 h postinoculation and then gradually increased again (except for a lack of *tppC* upregulation). *A. fumigatus* double trehalose synthase Δ*tpsA* Δ*tpsB* mutants were sensitive to heat shock: at 50°C, the majority of conidia were nonviable and viable spores had greatly delayed germination, growth, and sporulation (81). Trehalose is also involved in resistance against oxidative stress, as the Δ*tpsA* Δ*tpsB* mutants also had reduced viability after exposure to ROS (81). It is thought that trehalose biosynthesis is important for cell wall stability; in *A. fumigatus*, however, no direct effect between trehalose production and cell wall integrity was found (82). Thammahong et al. used immunoprecipitation assays coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) to reveal interaction between the trehalose-related regulatory protein TslA and CsmA, a class V chitin synthase. A Δ*tslA* strain had an altered cell wall structure which resulted in exposure of microbe-associated molecular patterns (MAMPs) (83). MAMPs are conserved microbe-specific molecules, for example, chitin, that are recognized by the host innate immune system. Decreased virulence was observed in Δ*tslA* strains compared with that of wild-type strains.

Accumulating mannitol, a compatible solute ubiquitous in the fungal kingdom, is another mechanism by which fungi endure adverse environmental conditions. Like trehalose, this compatible sugar accumulates in dormant conidia and is degraded during germination (Fig. 1) (80). In *A. fumigatus*, the MpdA protein was identified as highly abundant in dormant conidia by two different studies (38, 39). Additionally, transcripts of *mtdA* and *mpdA* were found abundantly present in dormant conidia of *A. niger* (30). The *mtdA* gene encodes a predicted mannitol dehydrogenase, and *mpdA* encodes mannitol 1-phosphate dehydrogenase, the first enzyme in the mannitol biosynthesis pathway. Like the trehalose biosynthesis genes, transcript levels of *mtdA* and *mpdA* strongly decreased and then gradually increased again during germination. In *A. niger*, conidia of a Δ*mpdA* strain were highly sensitive to adverse conditions such as oxidative stress and high temperatures (84). In *Neosartorya fischeri*, a species very closely related to *A. fumigatus*, a decrease in mannitol content (Δ*mpdA* strain) resulted in reduced resistance against high temperature and oxidative stress (85).

Heat Shock Proteins

HSPs are a family of intracellular proteins upregulated by a variety of different stressors (Fig. 1). These proteins were labeled HSPs because they were identified after heat stress. HSPs have a variety of different functions, but the majority of characterized HSPs act as molecular chaperones or as proteases (86). Other HSPs are involved in various biological processes, such as transcription, translation, and posttranslational modifications (87). Molecular chaperones form complexes with diverse protein sub-

strates to assist protein folding. HSPs play a major role in the recovery from cell stress by acting as catalytic enzymes assisting in the refolding of mismatched or aggregated proteins, as a result promoting cell survival (86, 88). Hsp90 is an ATP-dependent chaperone that interacts with multiple client proteins involved in resistance against stress and high temperatures (89). Genetic repression of *A. fumigatus hsp90* leads to a decreased hyphal growth, decreased spore viability, and serious defects in conidiation and germination. Additionally, conidiation-specific transcription factors WetA, AbaA, and BrlA were shown to be downregulated (90). Suh et al. used quantitative shotgun proteomics to study developmental-stage-specific proteins. They identified several other HSPs present in dormant conidia of *A. fumigatus* (Scf1, Awh11, BipA, Hsp70, ClxA, and Egd2) (38). Cagas et al. also characterized proteomic changes during the different developmental stages. They used a gel-free iTRAQ system to identify a total number of 461 proteins, of which 231 were identified with high confidence, in *A. fumigatus*. Hsp30 was shown to decrease 9.5-fold in isotropically swelling conidia compared with dormant conidia (37). van Leeuwen et al. found transcripts in dormant conidia of *A. niger* homologous to several HSPs (30). The gene An06g01610 encodes a protein homologous to Hsp9 of *Schizosaccharomyces pombe* and Hsp12 of *Saccharomyces cerevisiae*. In *S. cerevisiae*, this LEA (late embryogenesis abundant)-like protein has been shown to protect the plasma membrane against desiccation (91). Another gene found (An01g13350) encodes a homologue of Hsp104 of *S. cerevisiae*, which is of importance to thermotolerance, ethanol tolerance, and sodium arsenite tolerance (92). Together with trehalose, Hsp104 provides acquired resistance against heat stress (93). The putative HSPs An15g05410, An07g09990, and An18g00600 were also accumulated in dormant conidia (30). Tiwari et al. described in their review that HSPs in fungi play a role in morphogenesis and resistance against adverse conditions such as temperature, pH, and osmotic pressure (87).

The Conidial Cell Wall and Surface Proteins

The polysaccharide cell wall is a major characteristic of the fungal cell and is essential for growth as well as protection against environmental stresses (Fig. 1). The *Aspergillus* cell wall is almost exclusively composed of different polysaccharides (94). The fibrillar core of the cell wall is composed of a branched β-(1,3)-glucan to which chitin/chitosan, β-(1,3)-glucan, β-(1,4)-glucan, β-(1,5)-galacto-α-(1,2)-mannan, and β-(1,5)-galacto-α-(1,6)-mannan are covalently bound. The outer layer of dormant conidia is covered by a rodlet layer; this rodlet layer is exclusively composed of hydrophobic RodA proteins in *A. fumigatus*. RodA was found to be abundantly present in dormant conidia of *A. fumigatus* (37). Secreted hydrophobic RodA was also found in *A. nidulans* conidia (95). RodA is responsible for the integrity of the cell wall and phialide modification. The latter has impact on conidial production, survival, and sensitivity to external stresses like desiccation, physical damage, and drugs (96). Dormant conidia have a pigment layer beneath the surface rodlet layer. A biologically important pigment in many fungi is melanin. In fungi, two types of melanin are found, 1,8-dihydroxynaphthalene (DHN) melanin and L-3,4-dihyroxyphenylalanine (L-DOPA) melanin. DHN melanin is produced by polymerization of 1,8-dihydroxynaphthalene, whereas L-DOPA melanin is produced by polymerization of dihydroxyindoles (97).

In *A. fumigatus*, the pigment layer is formed by DHN melanin. This layer is important for the structure and stiffness of the dormant cell wall (98, 99). Abr2 and Arp1, two proteins involved in conidial pigment biosynthesis (100, 101), were among the highly expressed proteins in dormant *A. fumigatus* conidia (37, 38). Recently, using RNA-Seq, we found transcripts of *arp1*, *arp2*, and *ayg1* in dormant *A. fumigatus* conidia; these genes are important for melanin biosynthesis (29). Arp1 and Arp2 are involved in the DHN biosynthesis pathway in *A. fumigatus* (99). Transcripts of melanin biosynthesis pathway genes *mcoC* (strong similarity to *A. fumigatus abr2*), *fwnA*, and *ayg1* were also present in dormant conidia of *A. niger* and decreased during germination (30, 102). In *A. niger*, however, there is little evidence that supports the involvement of the DHN pathway, as the *A. niger* genome lacks orthologues for *arp1* and *arp2* and pigmentation

is resistant to the DHN biosynthesis pathway inhibitor tricyclazole (102). The pigment extracted from two highly melanized *A. nidulans* strains was very similar to synthetic L-DOPA melanin (103). Melanin and RodA hydrophobins form a dense outer layer of the conidial cell wall. Extracted RodA from conidia was immunologically inactive and failed to activate dendritic cells, alveolar macrophages, or helper T cell immune responses in mice (104). Additionally, removal of the RodA surface layer, either chemically, genetically, or biologically, resulted in immunostimulatory morphotypes (105). In the absence of RodA, β-(1,3)-glucan and α-mannose were exposed on the conidial cell wall (106). In the absence of RodA, the natural killer (NK) cell-receptor-like C-type lectin Dectin-1 and the C-type lectin-like receptor (CTLR) member Dectin-2 were induced. RodA hydrophobins are important for survival of conidia inside the host; interfering with this important layer could be an alternative strategy for therapy.

Asif et al. used two-dimensional gel electrophoresis (2-DE) and LC-MS/MS to study the conidial surface proteome (36). They identified 26 proteins, of which 12 proteins had a signal sequence for cellular secretion. Among these were the known important conidial surface protein RodA, endoprotease PEP2, a putative extracellular lipase (Afu5g02040), PdiA, and a putative phosphoglycerate mutase family protein (Afu7g06750). Among the other isolated proteins were seven putatively secreted proteins with unknown function (Afu1g11480, Afu5g07890, Afu1g13670, Afu6g04690, Afu8g00630, Afu6g14470, and Afu5g01420). Fourteen of the 26 identified proteins did not have a signal for secretion. Among these surface proteins were the known allergen Aspf3, CipC, a putative tropomyosin (Afu7g04210), a putative acyl coenzyme A (acyl-CoA) binding family protein (Afu2g11060), Hsp90 binding cochaperone Wos2, and 60S acidic ribosomal protein Rpp1. Six of the 14 proteins without a signal peptide had no specific function (Afu2g08190, Afu1g09890, Afu8g01980, Afu5g03750, Afu1g10260, and Afu1g02290). These proteins still have an unknown function. Some of these proteins could be important virulence factors or interesting therapeutic targets. Therefore, more studies analyzing protein structure and function are required to fully understand the conidial cell wall.

Recently, an extensive *A. fumigatus* conidial surface proteome analysis was carried out using LC-MS/MS (107). One protein identified was nearly as abundant as rodlet protein RodA and was designated conidial cell wall protein A (CcpA). The amphiphilic CcpA was produced during sporulation and detectable on the surface of resting conidia. Germination rate and radial growth were not affected in Δ*ccpA* mutants. In addition, Δ*ccpA* conidia showed no significant changes with respect to susceptibility to oxidative stress, cell wall/membrane stress, or temperature stress. However, Δ*ccpA* mutants were more susceptible to immune recognition and rapid clearing; consequently, virulence was severely attenuated. Infection studies using immunocompromised mice showed that CcpA was required for virulence, suggesting that the cell wall protein could be used as a possible immunotherapeutic or diagnostic target.

Dehydrin-Like Proteins

A group of proteins called dehydrins was first described for plants and has a protective role against various dehydration stress conditions (108). Dehydrin-like proteins were also detected in aspergilli (Fig. 1) (74, 109, 110). The first two dehydrin-like proteins, called DprA and DprB, were identified during a search for genes controlling conidial dormancy in *A. fumigatus* (74). The proteins contained a repeated domain of 23 amino acids, consisting of a conserved dehydrin-like protein (DPR) motif. Deletion mutants of *dprA* were hypersensitive to oxidative stress and to killing by lung phagocytes. Disruption of *dprB* resulted in mutants with impaired osmotic and pH stress responses. A third dehydrin-like gene, called *dprC*, was detected in the genome of *A. fumigatus*; Δ*dprC* mutants were impaired in freezing tolerance (109). These two studies reveal a novel family of stress-protective proteins in *A. fumigatus*. Wartenberg et al. aimed at profiling the proteomic stress response in *A. nidulans* upon exposure to the antifungal farnesol (110). One of the findings was the strong upregulation of a dehydrin-like protein, called DlpA, which contained the signature pattern of fungal

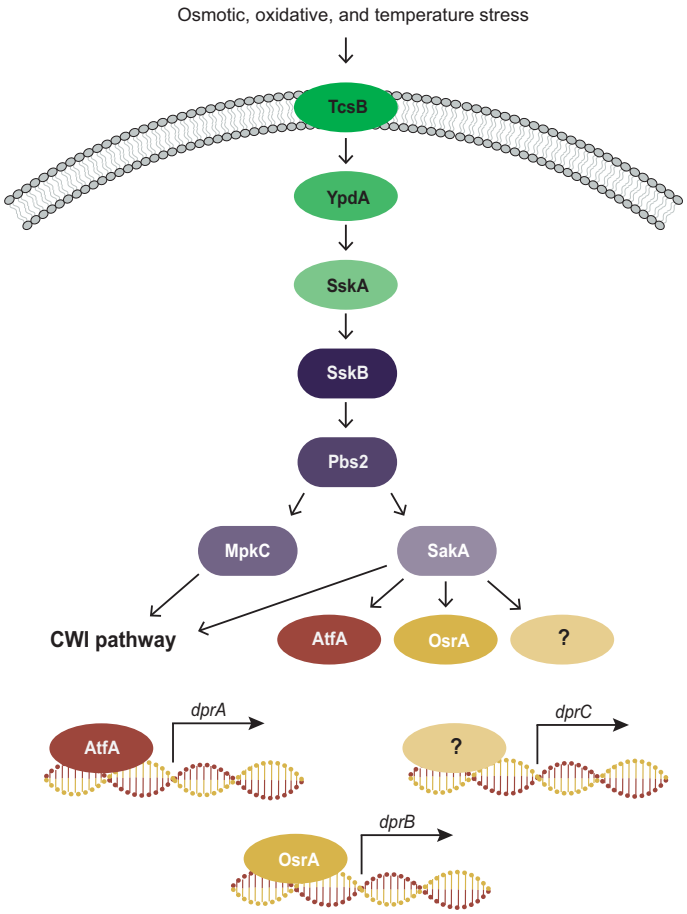

FIG 4 The HOG-MAPK pathway. The HOG-MAPK pathway is responsible for conidia stress tolerance. SskA is the final receptor of the TcsB-YpdA-SskA two-component signaling system and is the key activator of SskB (MAPKKK). Pbs2 (MAPKK) is activated by SskB. MpkC (MAPK) and SakA (MAPK) are the main effectors of the HOG pathway, activated by Pbs2. SakA and MpkC are involved in activating the CWI pathway. Transcription factor AtfA and the hypothetical osmotic stress regulator OsrA are activated by SakA. DprA and DprB are involved in the protection against oxidative, osmotic, and pH stress. DprC is involved in the protection against freeze stress.

dehydrins. Δ*dlpA* deletion mutants showed significantly increased susceptibility to osmotic stress as well as high temperatures. *dprA* mutants were hypersensitive to oxidative stress, which is a stressor inside the host, and to killing by phagocytes; therefore, exploring dehydrin-like proteins as targets for antifungal therapy might be promising.

The High-Osmolarity Glycerol–Mitogen-Activated Protein Kinase Pathway

Mitogen-activated protein kinase (MAPK) pathways respond to extracellular signals and transmit this information intracellularly, therefore regulating a variety of biological processes, including stress responses (111). The high-osmolarity glycerol (HOG)-MAPK pathway (HOG pathway) is responsible for stress tolerance and is activated in conidia to regulate stress responses (Fig. 4) (72, 112). Figure 4 is based on the knowledge from *A. nidulans* and *A. fumigatus* studies. The HOG pathway is activated by an osmosensing two-component signaling system consisting of the proteins TcsB, YpdA, and SskA in *A. nidulans* (113). SskA is the final receptor of the TcsB-YpdA-SskA two-component signaling system and is the key activator of MAPK kinase kinase (MAPKKK) SskB (113). The MAPK kinase (MAPKK) Pbs2 is activated by MAPKKK SskB. MAPKs MpkC and SakA are the main effectors of the HOG pathway, activated by the upstream MAPKK Pbs2 (72, 113). Δ*pbs2*, Δ*sakA*, and Δ*sakA* Δ*mpkC* mutants showed reduced trehalose content and were sensitive to heat, osmotic, and oxidative stresses and to cell wall damage, leading

to reduced spore viability in *A. fumigatus* (72, 114). In Δ*sakA* Δ*mpkC* mutants, the cell wall integrity (CWI) pathway was not activated; these mutants were more sensitive to cell wall-damaging agents (114). Like the HOG pathway, the CWI pathway is important to counteract environmental stress. Furthermore, SakA interacts with transcription factor AtfA and MpkC to regulate stress-related responses (115, 116). Dehydrin-like proteins DprA and DprB are involved in protection against oxidative, osmotic, and pH stresses (74). Transcription factor AtfA is activated by SakA. In an Δ*atfA* mutant, *dprA* expression was impaired, indicating that *dprA* acted downstream of SakA and AtfA (74). The expression of *dprB* was dependent on the HOG pathway and on the hypothetical osmotic stress regulator OsrA (74). DprC is involved in protection against freeze stress (109). In Δ*sakA* mutants, no transcripts of *dprC* were detected, indicating that DprC acted downstream of SakA. Additionally, the study showed activation of the HOG pathway upon cold stress (109). Itraconazole (ITZ) and amphotericin B (AMB) are both considered oxidative stress drugs. A Δ*sakA* mutant displayed increasing sensitivity to ITZ- and AMB-induced oxidative stress (117). The increased sensitivity could be explained by the role SakA plays in regulating *dprA* expression. The downstream dehydrin-like proteins could be potential therapeutic targets, likewise are the upstream regulators of the HOG pathway.

REGULATION OF CONIDIAL GERMINATION

Nutrient Sensing and Signaling Pathways

Initiation of conidial germination requires the sensing of external signals. In most filamentous fungi, conidial germination requires the presence of low-molecular-mass nutrients such as inorganic salts, sugars, and amino acids (118). For example, germination in *Neurospora crassa* occurs in the presence of a carbon source and salt (119), whereas the presence of a glucose source alone was sufficient for germination in *A. nidulans* (45). A heterotrimeric G-protein pathway is present as a carbon source sensor in conidia, and the cAMP-PKA signaling pathway is activated for early events of germination (120). Additionally, the activity of the Ras signaling pathway plays a role in carbon sensing and controls the switch from isotropic growth to polarized growth (45, 121).

G proteins for signal transduction (Fig. 5). G proteins are essential for signal transduction and are involved in numerous biological processes, such as growth, differentiation, sporulation, and metabolism (122). The signaling system consists of a G-protein-coupled receptor (GPCR), heterotrimeric G proteins formed of one α subunit (Gα), one β subunit (Gβ), one γ subunit (Gγ), and a number of effectors (123). In the inactive state, Gα binds GDP and forms a complex with Gβ and Gγ that is associated with the GPCR. Exchange of GDP for GTP on Gα leads to dissociation from Gβ and Gγ and the GPCR. Active Gα-ATP and G$\beta\gamma$ dimer both regulate downstream effector proteins for various biological processes (124). In *A. nidulans*, the G proteins GanB (Gα), SfaD (Gβ; AN0081), and GpgA (Gγ; AN2742) are required for proper germination of conidia (120, 125). The α subunit GanB plays a primary role in cAMP-PKA signaling in response to glucose, whereas the G$\beta\gamma$ subunit SfaD and GpaA heterodimer are required for proper activation of GanB (120). In Δ*ganB* mutants, swelling of conidia and the formation of a germ tube were both delayed (125). Constitutive activation of GanB allowed germination of conidia without the presence of a carbon source, indicating GanB plays an important role during the early events of conidial germination. In *A. fumigatus*, homologues of the above-mentioned G-protein subunits have been characterized, designated GpaB (Afu1g12930) (Gα), SfaD (Afu5g12210) (Gβ), and GpaA (Afu1g05210) (Gγ) (126, 127). *A. fumigatus* SfaD and GpaA were required for vegetative growth, conidium germination, and trehalose breakdown (127). The G-protein α subunit GpaB was identified in *A. flavus*; however, the role of GpaB in conidium germination was not investigated (128).

The cAMP-PKA pathway (Fig. 5). The cAMP-dependent PKA is a heterotetramer consisting of a dimer of regulatory subunits and two catalytic subunits. The glucose-stimulated activation of the cAMP-PKA pathway (PKA pathway) requires a functional

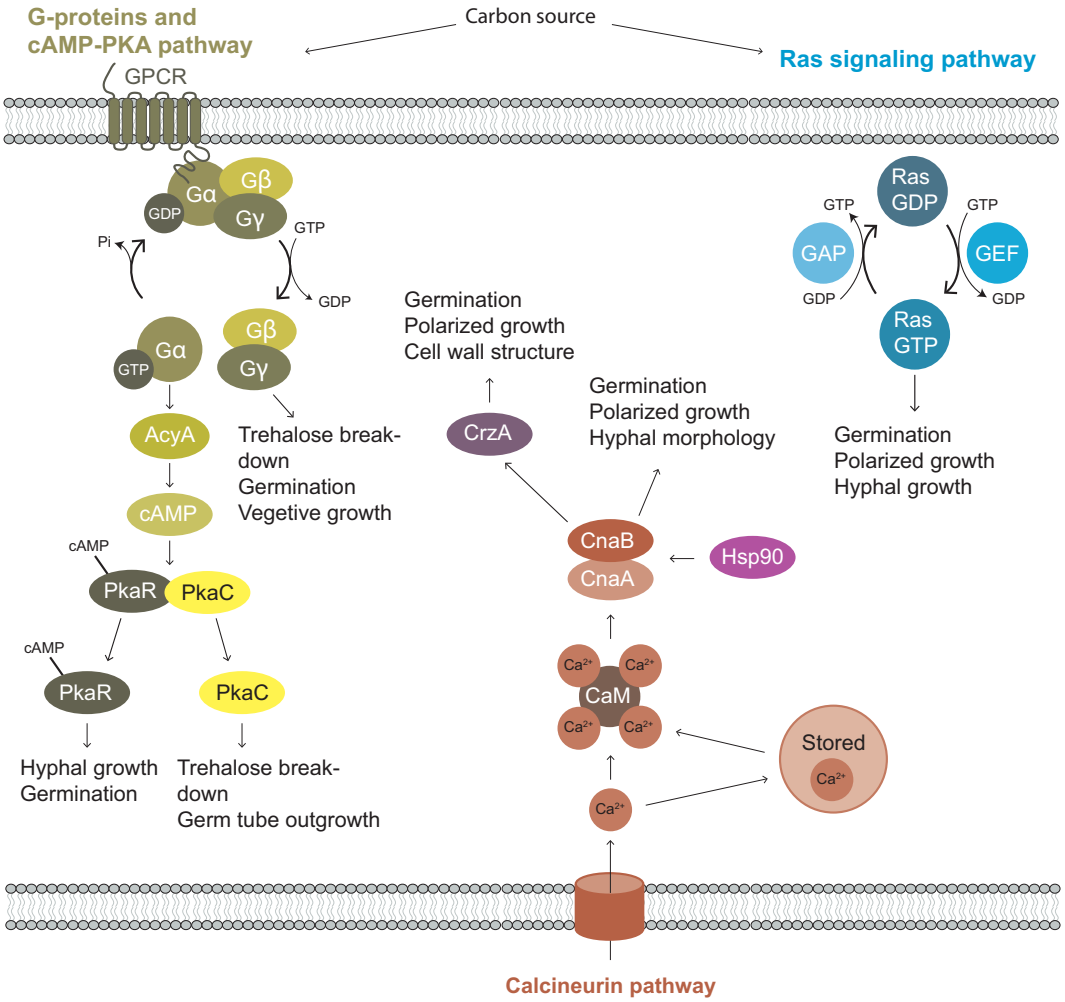

FIG 5 Regulatory pathways involved in conidial germination. The pathways shown are based on the knowledge from *A. nidulans* and *A. fumigatus* studies. G proteins are essential for signal transduction. Gα binds GDP and forms a complex with Gβ and Gγ that is associated with the GPCR. Exchange of GDP for GTP on Gα leads to dissociation from Gβ and Gγ and the GPCR. Gα-ATP and G$\beta\gamma$ dimer both regulate downstream effector proteins for various biological processes. AcyA is activated by Gα and produces cAMP, which binds to PKAR to release the regulatory subunit from the catalytic subunit. Active PkaC can now phosphorylate downstream targets. GEFs activate Ras by exchanging Ras-bound GDP for GTP. GAPs stimulate hydrolysis of Ras-bound GTP to GDP, thereby deactivating Ras. Calcineurin is a heterodimer composed of a regulatory subunit (CnaB) and a catalytic subunit (CnaA); the heterodimer is activated by Ca^{2+} and CaM. A downstream effector of calcineurin is the transcription factor CrzA. Hsp90 interacts with calcineurin, thereby orchestrating cell wall integrity and conidiation.

GanB, SfaD, and GpgA G protein (120). The components of the PKA signaling cascade are adenylate cyclase CyaA (AN2623, AcyA/Afu6g08520), second messenger cAMP, and PkaA (AN6305, PkaC/Afu2g12200) (126, 129). CyaA is activated by GanB and produces cAMP, which binds to the regulatory subunit of PKA (PKAR). When cAMP binds to PKAR, a conformational change occurs, which releases the regulatory subunit from the catalytic subunit (PKAC). This activates the primary catalytic subunit PkaA, which can now phosphorylate downstream targets. In *A. fumigatus* Δ*acyA* mutants, no cAMP levels were detected (129), and no trehalose breakdown was observed during the onset of germination in Δ*acyA* mutant conidia. Furthermore, germ tube outgrowth was affected. In *A. nidulans* Δ*pkaA* mutants, trehalose breakdown was reduced and germ tube outgrowth was affected; however, the observed reductions were not as strong as in the Δ*acyA* mutant (129). Inactivation of the *A. fumigatus* and *A. niger pkaC* homologues (Afu2g12200 and An02g04270, respectively) led to similar defects during conidial germination (126, 130). Additionally, an *A. fumigatus* Δ*pkaC* mutant showed hypersensitivity to the cell wall-targeting agent caspofungin, indicating that the PKA pathway

plays a role in the cell wall stress response (131). Deletion of the regulatory subunit *pkaR* in *A. fumigatus* resulted in a number of phenotypic defects, including reduced hyphal growth and germination (132). Conidia from a Δ*pkaR* mutant were more sensitive to killing by hydrogen peroxide and were less virulent in immunosuppressed mouse models. Unregulated PKA signaling caused a loss of virulence in *A. fumigatus*, probably because of the reduced resistance against oxidizing agents and reduced growth kinetics. The PKA pathway plays a role in germination events and is important for the response against cell wall-damaging agents. Impaired germination together with a hypersensitive cell wall might be an ideal outcome for therapeutics; however, cAMP-activated protein kinases are also present in humans, which may cause toxicity in the host.

Ras signaling pathway (Fig. 5). Ras proteins belong to a family of small monomeric GTPases and act as signal transducer between external stimuli and multiple cellular processes. The activity of Ras proteins is mediated via GDP and GTP binding (133). Guanine nucleotide exchange factors (GEFs) activate Ras by exchanging Ras-bound GDP for GTP. GTPase-activating proteins (GAPs) stimulate hydrolysis of Ras-bound GTP to GDP, thereby deactivating Ras (133). Two Ras proteins have been identified in *A. fumigatus* and *A. nidulans*, RasA (Afu5g11230, AN0182) and RasB (Afu2g07770, AN5832); both play important roles in several biological processes, including germination, polarized growth, and hyphal morphogenesis (121, 134–137). Expression of a dominant inactive form of RasA in *A. fumigatus* resulted in a reduced rate of conidial germination (134). In contrast, expression of a dominant inactive form of RasB delayed the initiation of germination but did not affect the germination rate (134). Both Δ*rasA* and Δ*rasB* mutants displayed defects in hyphal morphology and polarized growth (135). A dominant active form of RasA did not switch to polarized growth in *A. nidulans*; instead, conidia kept swelling (121). This suggests that high RasA activity prevents conidia from proceeding to polarized growth. Additionally, expression of dominant active RasA caused conidia to swell in the absence of a carbon source (45). A similar phenotype was observed in a Ras GAP (Δ*gapA*) mutant (AN4998) (45). These data suggest a role for RasA in carbon sensing during germination and that this is regulated by GapA. The dominant active form of RasA blocks polarized growth in the absence of a functional *acyA* gene, a homologue of *A. nidulans cyaA*, suggesting that RasA regulates germination independently of AcyA (129). Additionally, intracellular cAMP levels increased independently of RasA activation, suggesting that cAMP-PKA and Ras signaling independently control spore germination.

Other Signaling Pathways

The calcineurin pathway (Fig. 5). Calcineurin is a heterodimer composed of a regulatory subunit (CnaB) and a catalytic subunit (CnaA); activation of the heterodimer is Ca^{2+}/calmodulin (CaM) dependent (138). After the catalytic subunit CnaA binds to the regulatory subunit CnaB, CnaA is activated by Ca^{2+} and CaM (139). *A. fumigatus* Δ*cnaA* (Afu5g09360) mutants exhibited defective hyphal morphology related to polarized growth as well as attenuation of pathogenicity (140). To study the function of the regulatory and the catalytic subunit in *A. fumigatus*, single and double mutants were generated (141). The Δ*cnaB* (Afu6g04540) mutants showed a morphology indistinguishable from that of the Δ*cnaA* mutants, revealing that CnaB is necessary for calcineurin activity. The Δ*cnaA* Δ*cnaB* mutants had delayed germination and a greater growth defect. An important downstream effector of calcineurin may be the zinc finger transcription factor CrzA (Afu1g06900) (142). Δ*crzA* mutants had significant defects in germination, polarized hyphal growth, and cell wall structure, similar to Δ*cnaA* mutants. The activated calcineurin complex dephosphorylates CrzA and triggers translocation to the nucleus, where it regulates cell wall biosynthesis genes (143). Additionally, the activated calcineurin complex may also interact with cell wall proteins directly to regulate activity and cell wall homeostasis. A protein interacting with calcineurin is Hsp90, orchestrating cell wall integrity and conidiation (144). Genetic repression of *hsp90* in *A. fumigatus* caused a decrease in spore viability and severe defects in conidial

germination. Calcineurin inhibitors have been studied as new antifungals due to their specific mode of action; however, the currently available calcineurin inhibitors bind to immunophilins, resulting in host immunosuppression (145).

EARLY GERMINATION AND ISOTROPIC AND POLARIZED GROWTH

Synchronous Development of Germinating Conidia

Several studies have monitored the morphology of germinating conidia and have shown that the first 8 h of development were relatively synchronous (29–31, 33, 42, 146). During the first 90 min of conidial germination, no morphological changes were observed. At 2 and 4 h postinoculation, conidia were isotropically expanding until they had doubled their size. At 6 h postinoculation, the growth was directed to one side of the cell, resulting in the formation of a germ tube. At 8 h postinoculation, all conidia had short germ tubes. The germination process was characterized by two distinct morphological changes: (i) isotropically expanding conidia, also referred to as isotropic growth, and (ii) formation of a germ tube, also referred to as polarized growth. In *A. terreus*, identical morphotypes were observed; however, the duration of the germination process is different from that of *A. fumigatus*, *A. niger*, *A. flavus*, and *A. nidulans* (41). In conidia of *A. terreus*, no significant morphological change was observed during the first 9 h. After that, conidia isotropically expanded and were swelling at 10 to 11 h. Conidia continued to swell until 14 h, after which polarized growth was observed. After 16 h, 90% of conidia had a formed germ tube.

Breaking Dormancy

The cell wall of germinating conidia. The hyphal cell wall differs in composition from the conidial cell wall (Fig. 6) (147). The inner cell wall is composed of branched β-(1,3)-glucan to which galactomannan, chitin, and β-(1,3)-glucan/β-(1,4)-glucans are covalently bound. In the conidial cell wall, the inner layer is covered by a dense outer layer formed by α-glucans, melanin, and RodA hydrophobins. In the hyphal cell wall, the inner layer is covered by an amorphous alkali-soluble layer which is composed of α-glucans, galactomannan, and galactosaminogalactan (GAG). GAG suppresses host inflammatory responses partly through masking β-glucans from recognition by Dectin-1. Additionally, in two murine models of invasive aspergillosis GAG was essential for full virulence, establishing a role as a pivotal bifunctional virulence factor in the pathogenesis of invasive aspergillosis (148). During germination, the dense outer layer of the conidial cell is shed and rodlets are degraded by aspartic proteases, after which hyphal growth proceeds. However, depending on the environmental conditions, the cell wall may still contain melanin. In the hyphae of germinating conidia isolated from the lungs of immunocompromised mice, the *pksP* gene (Afu2g17600), essential for melanin biosynthesis, was expressed (149). The conidial and hyphal cell walls are exposed to numerous external stressors, including antifungals. Compounds that disrupt the cell wall, such as some antifungal drugs, could activate the CWI pathway, which is mediated by the MAPK pathway. The CWI pathway plays a major role in cell wall maintenance and energy consumption in cellular processes involved in hyphal growth and development (150).

Transcriptomic landscape at breaking of dormancy (Fig. 2). Conidial germination requires translation but not transcription. Protein synthesis inhibitor cycloheximide prevented isotropic growth in conidia, whereas inhibitors of DNA and RNA synthesis did not affect this process (45). Stored mRNAs were found in resting *A. nidulans* conidia, and 12 genes with stored transcripts were identified (151). Lamarre et al. found that 27% of genes (844/3,182) had transcripts in dormant *A. fumigatus* conidia (33). Several functions have been described for these transcripts. (i) It could be that these transcripts are required for spore metabolism during dormancy; in spores of *Saccharomyces cerevisiae*, ongoing transcription and translation are necessary for long-term survival (152). (ii) A part of the prepacked transcripts in the conidia might be conidiation transcripts that will decay over time during conidial aging without any effect on survival or germination (153); however, most transcripts present in *A. fumigatus* conidia re-

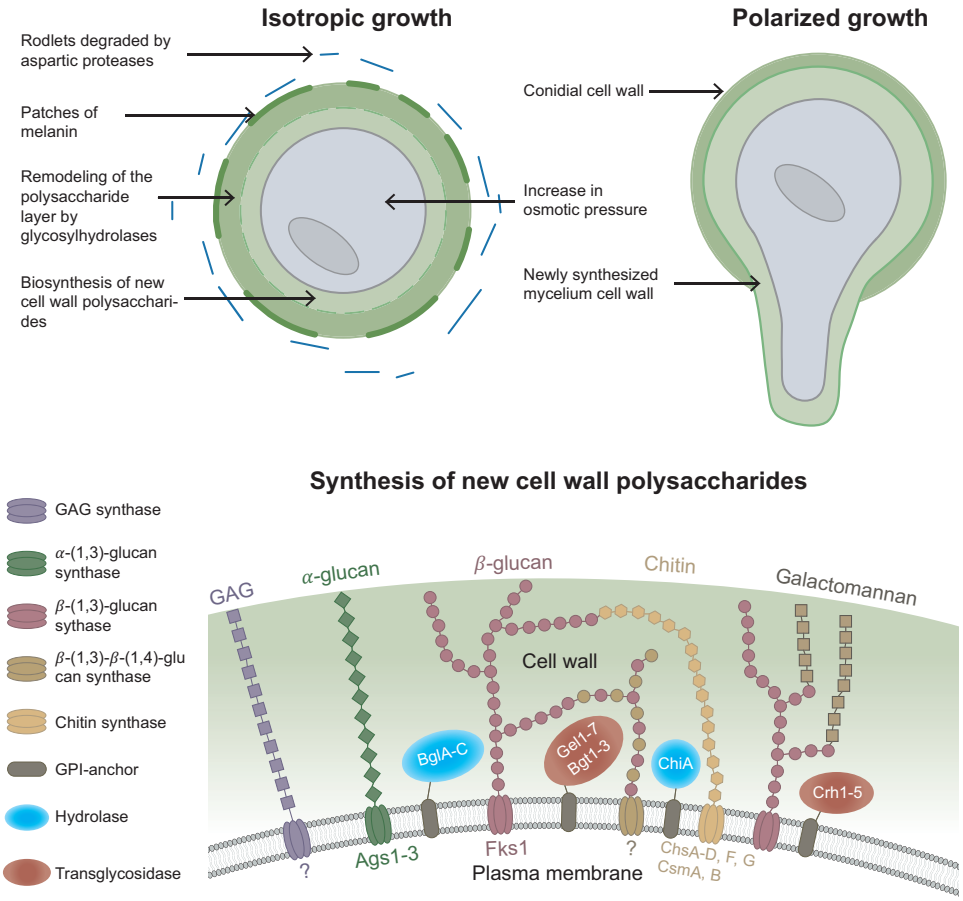

FIG 6 (Isotropic growth) Swelling of the conidium is the initial stage of the isotropic growth phase. Swelling is due to an increase in osmotic pressure as well as the modification of the conidial cell wall by glycosylhydrolases. The rodlet layer is degraded by aspartic proteases. (Polarized growth) The cell wall is modified, and biosynthesis of new polysaccharides leads to different polysaccharides on the surface of the cell wall, such as α-glucans, galactomannan, and galactosaminogalactan (GAG). (Synthesis of new cell wall polysaccharides) β-(1,3)-Glucan, β-(1,3)-β-(1,4)-glucan, chitin, α-(1,3)-glucan, galactomannan, and GAG are the main components of the cell wall. β-(1,3)-Glucan is synthesized by glucan synthase Fks1 and modified by Bgt1 to -3, Gel1 to -7, and glucanases BglA to -C. Chitin is synthesized by chitin synthases (ChsA to -D, -F, and -G and CsmA and -B) and modified by transglycosidases Crh1 to -5 and chitinase ChiA. α-(1,3)-Glucan is synthesized by Ags1 to -3 synthases.

mained there after 1 year of storage at room temperature (33). (iii) The dormant conidia are primed for rapid activation and translation when environmental conditions are suitable for germination; in *A. fumigatus*, protein synthesis is one of the earliest measurable biochemical changes during germination (154). Transcripts in dormant conidia corresponded to genes involved in translation regulatory activity, RNA binding, and alcohol and phosphorus metabolism. After breaking of dormancy (30 min postinoculation), genes belonging to fermentative metabolism and oxidoreductase activity were downregulated. Genes involved in RNA and phosphorus metabolism, the tricarboxylic acid (TCA) cycle, amino acid and protein synthesis, and protein complex assembly were upregulated (33). Lamarre et al. showed that breaking of dormancy is characterized by a shift from a fermentative metabolism to a respiratory metabolism and immediate protein synthesis (33). Teutschbein et al. detected pyruvate decarboxylase and alcohol dehydrogenase in dormant conidia, suggesting that fermentation plays a role in dormant conidia (39). Novodvorska et al. used RNA sequencing to study the transcriptional landscape at breaking of conidial dormancy (28). In dormant conidia of *A. niger*, transcripts of genes involved in gluconeogenesis, the glyoxylate cycle, and fermentation were found. The presence of these transcripts in dormant conidia indicates energy generation from noncarbohydrates for maintenance during dormancy.

Breaking of dormancy was associated with an increase of transcripts for genes involved in the biosynthesis of proteins, RNA turnover, and respiratory metabolism. This suggests, like in *A. fumigatus*, a switch from fermentative metabolism to respiratory metabolism at breaking of dormancy. More recently, however, Novodvorska et al. detected oxygen uptake and carbon dioxide generation in dormant conidia, suggesting a low-level respiratory metabolism for survival (27).

Isotropic Growth

Transcriptomic and proteomic landscape of isotropically swelling conidia (Fig. 2 and Table 2). The first morphological change in conidial germination is isotropic growth. This process involves water uptake and a decrease in the microviscosity of the cytoplasm (46). Conidia grow isotropically by adding new cell wall material equally in every direction (Fig. 6). At the end of this phase, the diameter of the cell is increased 2-fold or more (29, 30, 41). Suh et al. reported 215 proteins at 4 h after inoculation in *A. fumigatus*; 85 were identified as up-expressed in swelling conidia compared with dormant conidia at 0 h (38). The majority of these proteins were intracellular, and most of them were involved in protein synthesis and the TCA cycle. Apart from these intracellular proteins, five cell wall-associated proteins were also observed, including Ecm33, Bgt2, Gel1, and putative GPI-anchored cell wall protein Afu4g08960. Bgt2 is a β-(1,3)-glucanosyltransferase with β-(1,6)-branching activity. It cleaves laminaribose from the reducing end of a β-(1,3)-glucan chain and transfers the remaining β-(1,3)-glucan chain toward another glucan chain by a β-(1,6)-glucan linkage (155). *ecm33* encodes a GPI-anchored cell wall protein; disruption of *ecm33* results in an increased diameter of the conidia together with an increased concentration chitin in the cell wall. Conidia defective in *ecm33* were more resistant to killing by macrophages, whereas the mycelium was more easily killed by neutrophils (156). Phagocytic killing is dependent on reactive oxidants, and the higher susceptibility of mycelia could be associated with a higher susceptibility to reactive oxidants. The higher resistance of mutant conidia could be due to differences in cell wall organization. The mutant cell wall could have lower permeability to phagocyte toxic metabolites or lower sensitivity to host enzymes.

Transcriptomic analysis of germinating conidia showed an increase of transcripts involved in the remodeling of the cell wall during isotropic growth compared with the amount with dormant conidia (29). Transcripts of cell wall-associated proteins identified by Suh et al. (38) were also identified, such as *ecm33* and *gel1*. Other genes involved in the remodeling of the cell wall were also upregulated during isotropic growth; among these were *gel4* and *srb1*. *gel1* and *gel4* both encode 1,3-β-glucanosyltransferases with an important role in the elongation of 1,3-β-glucan chains. *srb1* encodes a GDP-mannose pyrophosphorylase which catalyzes the synthesis of GDP-mannose using mannose and GTP as substrates (157). In *A. niger*, an increase of transcripts coding for chitin synthases and GPI-anchored glucanosyltransferases was observed during isotropic growth, including *chsA*, *chsB*, *chsF*, *gelA*, *crhC*, and *crhD* (30).

Thus, these studies suggest that remodeling of the fungal cell wall is an important process during isotropic growth. The isotropically expanding conidia increase 2-fold in diameter. During this process the cell wall needs to extend, which is demonstrated by an increase in genes involved in the elongation of glucan chains. The rapidly expanding cell wall requires new material, like chitin, for its structural integrity. Like Suh et al. (38), Cagas et al. observed proteins involved in protein biosynthesis and TCA cycle in swelling conidia (37). Isotropically expanding of the conidia is concomitant with metabolic activities required for cellular growth, such as protein synthesis and carbohydrate metabolism.

Polarized Growth

Transcriptomic and proteomic landscape during polarized growth (Fig. 2 and Table 2). Isotropic growth is followed by polarized growth. During this phase, formation of a germ tube is observed. The model for polarized growth in filamentous fungi is that

TABLE 2 List of proteins and genes expressed during isotropic and polarized growth described in this review

Gene or protein	*Aspergillus* species	Systemic name	Description	Reference
Isotropic growth				
Gene				
gel4	*A. fumigatus*	Afu2g05340	Gene for an essential GPI-anchored 1,3-β-glucanosyltransferase with an important role in the elongation of 1,3-β-glucan chains.	29
srb1	*A. fumigatus*	Afu4g11510	Gene for GDP-mannose pyrophosphorylase which catalyzes the synthesis of GDP-mannose using mannose and GTP as substrates.	29
chsA	*A. niger*	An07g05570	Gene for class II chitin synthase involved in chitin biosynthesis.	30
chsB	*A. niger*	An09g04010	Gene for class III chitin synthase involved in chitin biosynthesis.	30
chsF	*A. niger*	An12g10380	Gene for class III chitin synthase involved in chitin biosynthesis.	30
gelA	*A. niger*	An10g00400	Gene for 1,3-β-glucanosyltransferase with an important role in the elongation of 1,3-β-glucan chains.	30
crhC	*A. niger*	An07g01160	Gene for putative GPI-anchored glucanosyltransferase with predicted role in glucan processing.	30
crhD	*A. niger*	An01g11010	Gene for putative GPI-anchored glucanosyltransferase with predicted role in glucan processing.	30
Protein				
Ecm33	*A. fumigatus*	Afu4g06820	GPI-anchored cell wall protein. Mutant conidia have an increased diameter together with an increased concentration chitin in the cell wall.	38
Bgt2	*A. fumigatus*	Afu3g00270	β-(1,3)-Glucanosyltransferase with β-(1,6)-branching activity. It cleaves and transfers the β-(1,3)-glucan chain towards another glucan chain by a β-(1,6)-glucan linkage.	38
Gel1	*A. fumigatus*	Afu2g01170	1,3-β-Glucanosyltransferases with an important role in the elongation of 1,3-β-glucan chains.	38
	A. fumigatus	Afu4g08960	GPI-anchored protein.	38
Polarized growth				
Gene				
chsA	*A. fumigatus*	Afu2g01870	Gene for putative class I chitin synthase involved in chitin biosynthesis.	29
chsF	*A. fumigatus*	Afu8g05630	Gene for putative class IV chitin synthase involved in chitin biosynthesis.	29
chsG	*A. fumigatus*	Afu3g14420	Gene for putative class III chitin synthase involved in chitin biosynthesis.	29
csmA	*A. fumigatus*	Afu2g13440	Gene for putative class V chitin synthase involved in chitin biosynthesis; required for normal hyphal growth.	29
csmB	*A. fumigatus*	Afu2g13430	Gene for putative class VI chitin synthase involved in chitin biosynthesis and hyphal tip growth.	29
aspA	*A. fumigatus*	Afu5g08540	Gene for septin involved in development.	29
aspB	*A. fumigatus*	Afu7g05370	Gene for putative septin; localizes to the septa during early septum formation.	29
aspC	*A. fumigatus*	Afu5g03080	Gene for putative septin involved in development.	29
aspD	*A. fumigatus*	Afu1g08850	Gene for septin; localizes to long tubular structures within hyphae and to newly formed septa.	29
gel2	*A. fumigatus*	Afu6g11390	Gene for GPI-anchored 1,3-β-glucanosyltransferase with a role in glucan processing.	29
gel5	*A. fumigatus*	Afu8g02130	Gene for putative 1,3-β-glucanosyltransferase with a role in glucan processing.	29
	A. fumigatus	Afu4g12160	Orthologue of *A. nidulans nimE*, encoding a mitosis-specific cyclin required for transition from the G_2 to the M phase of the cell cycle.	29
	A. fumigatus	Afu7g01400	Orthologue of *A. nidulans bimC*, encoding a kinesin-family protein required for separation of mitotic spindle bodies and important for normal completion of mitosis.	29
	A. fumigatus	Afu7g03890	Protein encoded by orthologue(s) has ATPase activity and a role in attachment of mitotic spindle microtubules to kinetochore.	29

(Continued on next page)

TABLE 2 (Continued)

Gene or protein	*Aspergillus* species	Systemic name	Description	Reference
eb1	*A. fumigatus*	Afu3g11860	Gene for putative microtubule-associated protein.	29
	A. fumigatus	Afu1g13390	Orthologue of *A. nidulans mipC*, encoding a gamma-tubulin essential for microtubule function in general and nuclear division in particular.	29
	A. fumigatus	Afu3g09370	Protein encoded by orthologue(s) has gamma-tubulin binding activity.	29
chiA1	*A. fumigatus*	Afu5g03760	Gene for putative class III chitinase with predicted role in chitin hydrolysis.	31
pfy1	*A. fumigatus*	Afu4g03050	Gene for putative acting-binding protein profilin.	31
	A. fumigatus	Afu1g05790	Gene for putative GPI-anchored protein.	31
	A. fumigatus	Afu2g07800	Gene for putative GPI-anchored cell wall protein.	31
	A. fumigatus	Afu5g11380	Orthologue of *S. cerevisiae* Rho-GDP dissociation inhibitor *rdi1* (YDL135C), involved in the localization and regulation of Cdc42 and Rho1.	31
rhoA	*A. niger*	An18g05980	Gene for Rho family GTPase essential for polarity establishment viability.	179
rhoB	*A. niger*	An16g04200	Gene for putative Rho-like GTPase with an important role in cell wall integrity and septum formation.	179
rhoC	*A. niger*	An11g09620	Gene for Rho GTPase.	179
rhoD	*A. niger*	An14g0553	Gene for putative Rho-like GTPase with an important role in cell wall integrity and septum formation.	179
racA	*A. niger*	An11g10030	Gene for Rho GTPase with a role in actin organization and polarity maintenance.	179
cftA	*A. niger*	An02g14200	Gene for Rho GTPase with a role in actin organization and polarity maintenance.	179
Protein				
HcsA	*A. fumigatus*	Afu4g10460	Homocitrate synthase, essential enzyme of the alpha-aminoadipate pathway of lysine biosynthesis.	38
SodA	*A. fumigatus*	Afu5g09240	Cu/Zn superoxide dismutase.	38
	A. fumigatus	Afu6g08360	Thiazole biosynthesis enzyme.	38
BtgE	*A. fumigatus*	Afu8g05610	Putative β-glucosidase with predicted role in glucan degradation.	38
PyroA	*A. fumigatus*	Afu5g08090	Key enzyme in the biosynthetic pathway of pyridoxine.	38
	A. fumigatus	Afu4g07710	Putative pyruvate carboxylase.	38
FksA	*A. flavus*	NA	β-1,3-Glucan synthase with a role in glucan biosynthesis.	42
ArgJ	*A. flavus*	AFLA_061910	Arginine biosynthesis bifunctional protein.	42
ChsC	*A. flavus*	NA	Class I chitin synthase with a role in chitin biosynthesis.	42
PgxC	*A. flavus*	AFLA_086360	Putative exopolygalacturonase specific in hydrolyzing the terminal glycosidic bond of polygalacturonic acid and oligogalacturonates.	42
Mcd4	*A. flavus*	AFLA_041530	GPI-anchored ethanolamine phosphate transferase I.	42
PlyA	*A. flavus*	AFLA_057770	Putative pectate lyase A.	42
BglA	*A. flavus*	AFLA_051140	Putative β-glucosidase involved in degradation of glucans.	42
BglB	*A. flavus*	NA	Putative β-glucosidase involved in degradation of glucans.	42
BglC	*A. flavus*	AFLA_138140	Putative β-glucosidase involved in degradation of glucans.	42
CgrA	*A. terreus*	ATEG_10388	Nucleolar rRNA-processing protein.	41
MyoA	*A. terreus*	ATEG_07759	Type I myosin implicated in the organization of the actin cytoskeleton; required for proper actin cytoskeleton polarization.	41
Mep	*A. terreus*	ATEG_07544	Secreted metalloproteinase that allows assimilation of proteinaceous substrates.	41
Hog1	*A. terreus*	ATEG_00489	Putative MAPK with predicted roles in the osmotic and oxidative stress responses.	41
MpkC	*A. terreus*	ATEG_06557	Putative MAPK involved in the oxidative stress response; transcript abundance increases in response to carbon source and oxidative stress.	41

swelling conidia first establish an axis of polarity and deposit cortical markers. Subsequently, polarity components such as the Cdc42 module and polarisome are recruited to the cortical markers. Eventually, the morphogenetic machinery, composed of actin, septins, cell wall biosynthetic enzymes, the vesicle trafficking system, signaling pathways, landmark proteins, Arp2/3 complexes, polarisome, and Rho GTPase modules, is directed to the site of polarization to add new cell material (158–163).

In our recent study, we analyzed the transcriptomes during isotropic and polarized growth. At 6 h, when polarized growth was initiated, expression of genes involved in hyphal growth was increased (29). Among these genes were genes for several chitin synthases (*chsA*, *chsF*, *chsG*, *csmA* [previously *chsE*], and *csmB*), septins (*aspA*, *aspB*, *aspC*, and *aspD*), and 1,3-β-glucanosyltransferases (*gel2* and *gel5*). Figure 6 shows the cell wall components and proteins involved in cell wall biosynthesis identified by the referenced transcriptomic and proteomic studies. Chitin is one of the major components of the fungal cell wall and is important for the shape and mechanical strength of the fungal cell. Chitin synthesis occurs at the plasma membrane with the release of the growing chitin chain into the polysaccharide layer. CsmA and CsmB are two chitin synthases with a myosin motor-like domain (MMD). The Δ*csmA* Δ*csmB* mutants did not show any modifications in the cell wall chitin content (164). Even though *csmA* and *csmB* contribute lightly to the overall chitin biosynthesis, resting and germinating conidia of a Δ*csmA* Δ*csmB* mutant were more susceptible to all echinocandins, which are inhibitors of β-(1,3)-glucan synthesis. However, no difference was found in enzymatic activity of the β-(1,3)-glucan synthases in the Δ*csm* mutants compared with that of the parental strain. In *A. nidulans*, CsmA and CsmB localize at the hyphal tip and forming septa, where they perform compensatory functions that are essential for hyphal tip formation (165). The MMD of both CsmA and CsmB functioned as an anchor to bind to actin filaments *in vitro* (165, 166); this interaction was important for proper localization and functioning of CsmA (166). The continuous movement of chitin synthases to hyphal tips is necessary for hyphal tip growth in filamentous fungi. Actin-based and microtubule-based motors are both involved in this process (167).

An increase in transcript levels was also observed for genes involved in the cell cycle and DNA processing (29). Among those genes were Afu4g12160, Afu7g01400, Afu7g03890, *eb1*, Afu1g13390, and Afu3g09370. Afu4g12160 is an orthologue of *A. nidulans nimE*, which encodes a mitosis-specific cyclin required for transition from the G_2 to the M phase of the cell cycle (168). Afu7g01400 is an orthologue of *A. nidulans bimC*, which encodes a kinesin-family protein required for separation of mitotic spindle bodies and important for normal completion of mitosis (169). Afu1g13390 is an orthologue of *A. nidulans mipC*, which is a gamma-tubulin essential for microtubule function in general and nuclear division in particular (170, 171). Multiple nuclei were observed after 6 h of germination, which was coordinated with the expression of microtubule-associated genes (29). Conidia prepare for mitosis as is demonstrated by an increased expression of genes involved in the cell cycle. Microtubules are also essential for many cytoskeletal functions and are one of the main cytoskeleton components together with actin, motors, associated proteins, and septins (161). The cytoskeleton controls organelle positioning and movement, maintains cytoplasmic organization, and plays an important role in tip growth and hyphal morphogenesis (172). Phenotypic and transcriptomic analyses showed that nuclear division and the formation of a germ tube were coordinated in germinating *A. fumigatus* conidia (29).

Oda et al. found that 46 genes were significantly upregulated during the switch from isotropic to polarized growth (31). The most highly upregulated genes were associated with the cytoskeleton and cell wall and are possibly involved in the remodeling of cell morphology. Highly expressed genes include putative chitinase gene *chiA1*, actin-binding protein profilin gene *pfy1*, putative GPI-anchored protein gene Afu1g05790, putative GPI-anchored cell wall protein gene Afu2g07800, and the gene for the putative Rho-GDP dissociation inhibitor Afu5g11380. The gene for Afu5g11380 is an orthologue of *S. cerevisiae* Rho-GDP dissociation inhibitor gene *rdi1* (YDL135C) and is involved in the localization and regulation of Cdc42 and Rho1 (173). In *S. cerevisiae*, the small rho-type GTPase Cdc42 (YLR229C) is essential for establishment and maintenance of cell polarity and the GTP-binding protein Rho1 (YPR165W) is involved in establishment of cell polarity (174–177). The small monomeric Rho GTPases function as molecular switches to regulate various cellular processes such as morphogenesis, survival, vesicle transport, and differentiation (178). Most Rho GTPases cycle between an active GTP-bound state and an inactive GDP-bound state,

which is controlled by GEFs (exchange GDP for GTP) and GAPs (facilitate conversion of bound GTP to GDP) (178). Six Rho GTPases were identified in *A. niger*, which were designated *rhoA*, *rhoB*, *rhoC*, *rhoD*, *racA*, and *cftA* (179). RhoA is essential for polarity establishment and performs functions essential for viability and RhoB and RhoD play an important role in cell wall integrity and septum formation, whereas RhoC seems dispensable for *A. niger*. RacA plays a role in actin organization, localizes to the hyphal tip, and controls polarity maintenance. RacA and CftA (Cdc42 in *S. cerevisiae*) share overlapping functions in which RacA appeared to be more important for maintaining hyphal polarity. The Rho GTPases have critical roles during hyphal morphogenesis and could therefore be attractive targets for therapy. RhoA is essential for *A. niger* and *A. nidulans* and performs critical functions during polarity establishment and maintenance (179, 180). The *A. niger* Δ*rhoA* heterokaryotic mutant was defective in outgrowth of germ tubes and was characterized by swollen conidia (179).

Suh et al. identified 127 proteins overexpressed during germ tube formation compared with dormant conidia of *A. fumigatus* (38). Some of the overexpressed proteins may have an important role during establishment of infection, as its transcripts were upregulated during initiation of murine infection (181). Among those proteins were homocitrate synthase HcsA, involved in lysine biosynthesis, Cu/Zn dismutase SodA, which is induced by hydrogen peroxide, putative thiazole biosynthesis enzyme Afu6g08360, putative cell wall glucanase BtgE, putative pyridoxine biosynthesis protein PyroA, and putative pyruvate carboxylase Afu4g07710 (38). *pyroA* encodes a key enzyme in the biosynthetic pathway of pyridoxine (vitamin B_6). Deletion of *pyroA* in *A. fumigatus* resulted in attenuated virulence in murine infection models (182). Functional characterization of the other proteins is required to confirm their possible role as virulence factors. These targets could be exploited to make the fungus more susceptible to the host immune system.

Tiwari et al. analyzed the proteome of *A. flavus* at polarized growth (42). In total, 416 proteins were identified and categorized on the basis of their assigned Gene Ontology (GO) functional classification. Several cell wall-regulating proteins were identified; these proteins were β-1,3-glucan synthase FksA, arginine biosynthesis bifunctional protein ArgJ, class I chitin synthase ChsC, putative exopolygalacturonase PgxC, GPI ethanolamine phosphate transferase I Mcd4, and putative pectate lyase PlyA. Additionally, various glucan-degrading enzymes were also identified, such as putative β-glucosidases BglA, BglB, and BglC (42). Together with other studies, this shows that cell wall biogenesis is a major process during polarized growth (29, 38, 42). Polysaccharides such as α- and β-(1,3) glucans, galactomannans, and chitin are important structural components of the cell walls of *Aspergillus* species and interact directly with host cells. Proteins and enzymes involved in cell wall biogenesis are important components to focus on as antifungal targets and as diagnostic molecules. For example, the second most abundant polysaccharide is chitin, and in *A. fumigatus*, the above-mentioned chitin synthases CsmA and CsmB are essential for normal hyphal growth (164). It is of importance to further elucidate the components of the cell wall biosynthetic pathway and signal transduction, as those are promising targets for antifungals (183).

To identify proteins expressed during polarized growth in *A. terreus*, proteins were extracted after 16 h of growth and analyzed by LC-MS/MS (41). A total of 373 proteins were identified specific to *A. terreus*, of which 74 were uncharacterized. The Gene Ontology functions were assigned using UniProt. Proteins involved in RNA processing, protein transport, carbohydrate metabolism, transcription/regulation, protein biosynthesis, cell wall organization, DNA replication/repair, amino acid biosynthesis, and the cell cycle were identified. Besides these biological processes identified earlier in *Aspergillus* species, five probable virulence factors were identified. These five proteins were reported as virulence factors in *A. fumigatus* (114, 184–186). These were rRNA processing protein CgrA, type 1 myosin MyoA, extracellular metalloproteinase Mep, mitogen-activated protein kinase Hog1, and mitogen-activated protein kinase MpkC. In *A. fumigatus*, MpkC and SakA (Hog1)

were characterized as key virulence factors (114). The Δ*mpkC* and Δ*sakA* mutants had 40% reduction in fungal burden in mouse experiments. The double Δ*mpkC* Δ*sakA* mutants showed considerably attenuated virulence: approximately 50% of mice survived, and a fungal burden reduction of 75% was shown. The current arsenal of antifungal drugs to treat aspergillosis is limited, and emergence of resistance is a problem. New therapeutic strategies are desperately needed. Antivirulence approaches could be applicable to tackle this problem.

Sterol-rich membrane domains. Sterol-rich domains are important for establishment of polarity by allowing clustering of signaling and morphogenetic proteins within specific sterol-rich domains, also called lipid rafts (187, 188). Sphingolipids are major structural components of eukaryotic plasma membranes. Inhibition of the sphingolipid biosynthesis pathway prevented polarized growth in *A. nidulans*. Additionally, inhibition of the pathway after polarity establishment resulted in rapidly abolished cell polarity and promoted cell tip branching (189). Sphingolipids are required for localization of SepA to the hyphal tip (190). SepA plays an important role in the establishment and maintenance of polarity at hyphal tips (191). Actin filaments are required for proper locatization of SepA at septation sites and hyphal tips (192). In *A. fumigatus*, inhibition of sphingolipid biosynthesis resulted in sporulation and hyphal growth defects, leading to attenuated virulence (193). In a screen for drug compounds that target the biosynthesis of fungal sphingolipids, but not mammalian, two compounds [*N*′-(3-bromo-4-hydroxybenzylidene)-2-methylbenzohydrazide (BHBM) and its derivative, 3-bromo-*N*′-(3-bromo-4-hydroxybenzylidene) benzohydrazide (D0)] were found to be highly effective against several pathogenic fungi, including *Cryptococcus neoformans*, *Cryptococcus gattii*, *Rhizopus oryzae*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Pneumocystis murina*, and *Pneumocystis jirovecii* (194). However, activity was moderate against *Candida krusei*, *Candida glabrata*, *Candida guilliermondii*, *Candida parapsilosis*, *A. fumigatus*, and *Coccidioides* species. In a more recent study, antifungal activities of 19 derivatives of BHBM against several fungi were tested. One of them, called D13, improved the efficacy of itraconazole and voriconazole against *A. fumigatus* and was less toxic in mice (195). This new class of antifungals (acylhydrazones) showed promising results in terms of efficacy and toxicity.

CONCLUSION AND FUTURE CONSIDERATIONS

We have reviewed genes and proteins involved during the different morphological stages of germination and proposed potential targets for intervention. The key morphotypes are (i) dormancy, (ii) isotropic growth, and (iii) polarized growth. Dormant conidia are highly resistant against adverse environmental conditions. Regulatory pathways such as the cAMP-PKA and Ras pathway are involved in carbon sensing and play an important role in early events of germination.

Melanin and RodA hydrophobins are important for spore survival inside the host, and this dense outer layer could be attractive as a target for intervention. The HOG pathway is activated upon stress in conidia. The main effectors of this pathway are the protein kinases SakA and MpkC, which both activate transcription factors important for conidial survival. Both kinases are also involved in proper activation of the CWI pathway (114). The roles SakA and MpkC play in regulating stress responses and conidial viability make them interesting targets. Transcription factor AtfA is activated by SakA and regulates stress protection-related genes. Another transcription factor, MybA, potentially regulates *velvet* regulators *vosA* and *velB*. The VelB-VosA heterodimer is involved in trehalose biogenesis, cell wall integrity, and several stress responses, which makes this complex important for spore survival and viability. These transcription factors are activated by important stress- or germination-related pathways. Like these pathways themselves, important regulatory proteins are attractive targets for intervention.

The cell wall is constantly being remodeled and new material is added; genes involved in these processes could be promising antifungal targets. In *A. fumigatus*, a Δ*gel2* mutant exhibits slower growth, abnormal conidiogenesis, and altered cell wall composition (196). Another GEL family member, *gel4*, is essential for *A. fumigatus* (197). Deletion of *csmA* and

csmB, two chitin synthase genes, resulted in significant disorganization of the cell wall structure, leading to an increased sensitivity to echinocandin antifungals in *A. fumigatus* (164).

Using transcriptome and proteome analysis on specific *Aspergillus* morphotypes may further improve our understanding of the development and growth of different morphotypes. Germination is the key process required for conidia to form hyphae and cause disease. Identifying novel compounds essential for germination could provide opportunities for novel therapeutic strategies. Transcriptomics and proteomics of germinating *Aspergillus* conidia in mouse models or with host immune cells may provide insights into molecular events during pathogenesis (198, 199). RNA-Seq and proteome analysis could be extremely useful in such studies to unravel unknown mechanisms. Overall, fungal development and *Aspergillus* pathogenesis are important processes, and effort is needed to discover novel therapeutic targets, virulence factors, or biomarkers for early diagnosis.

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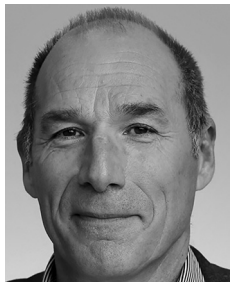

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