

Aus dem Institut für Klinische Biochemie
der Medizinischen Hochschule Hannover

Biosynthesis and Function of *Aspergillus fumigatus* Zwitterionic Glycolipid (Af-3c)

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„Biosynthesis and Function of *Aspergillus fumigatus* Zwitterionic Glycolipid (Af-3c)“

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Zusammenfassung (Dt.)

Die vorliegende Monographie beschreibt die experimentelle Annäherung an die Charakterisierung eines untypischen, zwitterionischen Glykolipids des opportunistisch humanpathogenen Fungus *Aspergillus fumigatus*. Dieses Glykolipid – Man- α -(1,3)-Man- α -(1,6)-GlcN- α -(1,2)-IPC – wird im folgenden Af-3c genannt. Glykoinositolphosphoceramide, die, wie das Vorliegende, eine GlcN- α -(1,2)-IPC-Kernstruktur besitzen, konnten interessanterweise bis heute nur in pathogenen Pilzen nachgewiesen werden und dies wirft die Frage auf, ob die Struktur in die Pathogenität involviert ist.

Das Gen-Cluster, das für die Expression des Af-3c synthetisierenden Enzymsystems verantwortlich ist, ist in vorangegangenen Studien identifiziert worden, ebenso die Lokalisation der meisten beteiligten Enzyme im Golgi-Apparat. Jedoch war eine Synthese von Af-3c allein durch die Expression des isolierten Enzymsystems in früheren Versuchen nicht erfolgreich, sodass wir in dieser Arbeit der Vermutung folgten, dass *in vivo* ein noch unidentifizierter Nukleotidzucker-Transporter für die Substratverfügbarkeit von UDP-GlcNAc (Uridindiphosphat-N-Acetylglucosamin) garantieren würde. Ein passendes Gen, das sich in Nachbarschaft zum betreffenden Gen-Cluster befindet, konnten wir im Genom von *A. fumigatus* identifizieren und klonieren.

Ziel der Arbeit war somit zum einen die Charakterisierung eines Nukleotidzucker-Transporters, aber auch die Analyse der Bedeutung von Af-3c in der Virulenz von *A. fumigatus*.

Dem vermeintlichen Transporter konnten wir uns durch Anwendung unterschiedlicher Methoden nähern, wie cDNA-Synthese, Sequenzierung, Klonierung, rekombinante Proteinexpression, spektrometrische Immunfluoreszenzdetektion, Golgi-Präparation und Transport-Assay mittels radioaktiv gelabelten Substrats, und konnten die Funktion des als UGT bezeichneten Proteins als UDP-GlcNAc-Transporter und auch seine Lokalisation *in vivo* im Golgi nachweisen.

Im zweiten Teil der Arbeit widmeten wir uns der Bedeutung von Af-3c für die *A. fumigatus* Pathogenität. Verschiedene Mutanten konnten mittels Kultivierung und durch Massenspektrometrie der Glykolipidprofile identifiziert und phänotypisiert werden. Durch eine experimentelle Reihe etablierte ich ein Protokoll zur Zellinfektion mit *A. fumigatus*-Sporen. Daraufhin konnte in einem Epithelzellmodell die Zellsterblichkeit des Wildtyps gegenüber der zur Af-3c-Synthese inkompetenten Mutante gegenübergestellt werden. Erste Ergebnisse zeigten eine verminderte Zellsterblichkeit derjenigen Zellkulturen, die mit Sporen der Mutante infiziert wurden, gegenüber den Wildtyp-infizierten Zellen.

Sowohl die Expression des gesamten für die Af-3c-Synthese verantwortlichen Gen-Clusters, wie auch die Konsolidierung der Ergebnisse im Zellmodell und die Ausweitung dessen auf weitere Mutanten können weiterführende Schritte in dieser Studie darstellen.

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1. INTRODUCTION

1.1 Introduction

Before bone marrow transplantation, steroid therapy and other immunocompromising situations were introduced in modern medicine, *Aspergillus fumigatus* (*A. fumigatus*) infections were not a subject of general medical and scientific interest.

The filamentous fungus *A. fumigatus* is an opportunistic pathogenic organism that, depending on the circumstances, can have a serious impact on humans' health. It is rather harmless for immunocompetent individuals and often detected in ambient air. But in immunosuppressed humans, this agent may cause aspergillosis, an acute or chronic pathological manifestation that can affect the lung or even have a systemic impact as invasive mycosis, which is difficult to combat and presents a high mortality rate. For example after stem cell transplantation, the mortality rate of an aspergillosis infection mounts up to 70 – 90% (1).

1.2 *Aspergillus* species

Aspergillus is a fungal genus including more than 339 species (2). Among these, the species *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus fumigatus* are human pathogens. Infections by *A. fumigatus* are however the most frequent and account for approximately 90% of hyphomycosis of inner organs in humans (1,3).

1.3 Microbiology

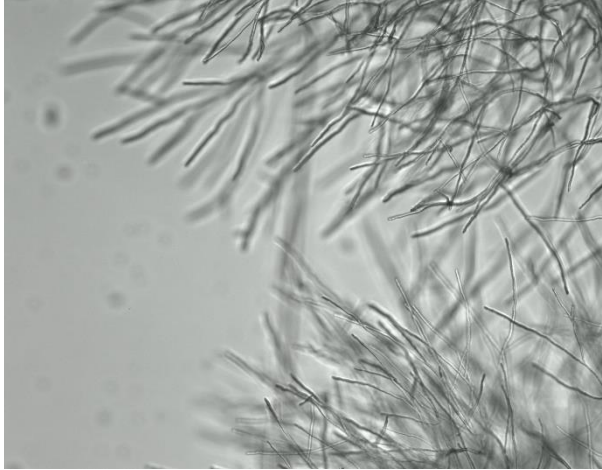
Fungi have a cell wall, built up of polysaccharides, like glucans, chitin and mannan, that guarantee mechanical and osmotic stability, as well as rigidity, which eases invasion in the host tissue (4). The cell wall encases the cell membrane with its main components, phospholipids, sterols and sphingolipids.

Aspergillus species belong to the division of Ascomycota, one of the largest phylum of fungi. This phylum includes yeasts and filamentous fungi. *A. fumigatus* is a saprophyte that grows in the soil fermenting decaying organic material. It reproduces asexually namely by the generation of small and light-weighted spores that are spread widely. The latter, also called conidia, can be found ubiquitously (5). *A. fumigatus* is highly acidotolerant and thermotolerant, it can even grow under pH 2 and at temperatures up to 55°C (1). *A. fumigatus* spores even survive temperatures around 70°C. The optimal temperature for *A. fumigatus* growth is 37°C, which is also the core temperature of endotherms.

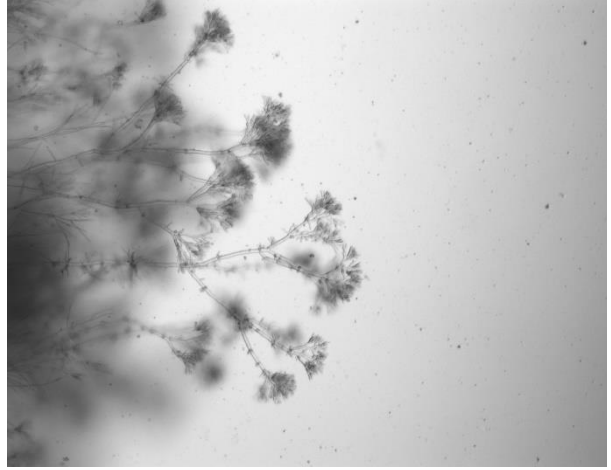
When getting in contact with air and humidity, the conidia rise to germ tubes and develop to septed hyphae, the macroscopically visible form is the whitish mycelium (see figure 1). They are characterised by outer brown-greenish stipes with oval heads, the phialides cells, which form the conidiophores (6). Conidia form at the tip of these conidiophores. When mature, they detach, and dispersal takes place by air circulation. If the environment or host cell offers adequate conditions, like nutrients, water, nitrogen,

carbon and trace elements, the conidia can germinate (7). Otherwise they can hibernate for months to years in spore status until environmental conditions turn more favourable.

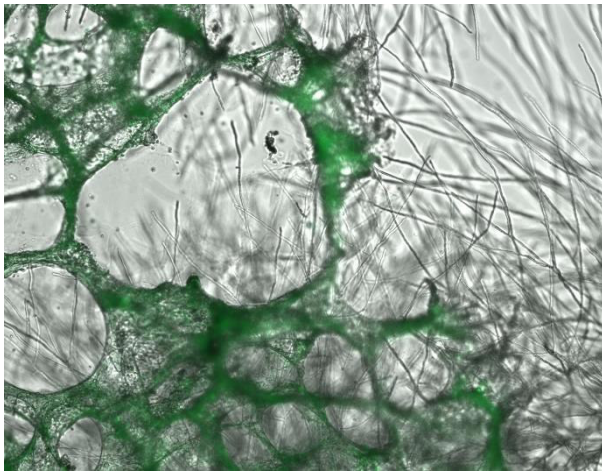
a.



b.



c.



- a. *A. fumigatus* hyphae after 48 h growth.
- b. Sporulation starting from the tips of the conidiophore after 24 h growth.
- c. Hyphae growth on lung epithelia within alveols *in vitro* on human precision cut lung slices after infection with *A. fumigatus* after 24 h growth (prestained with Calcein AM life staining)

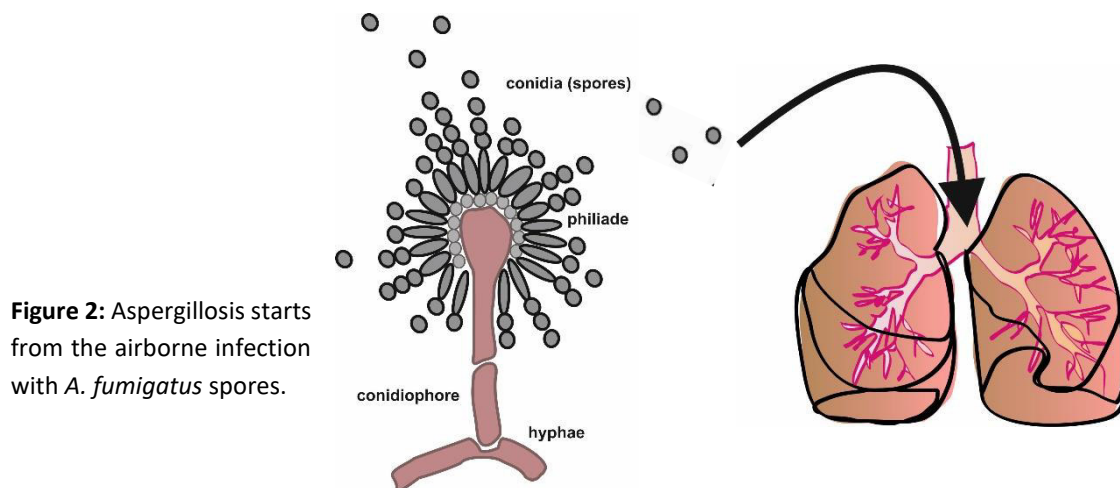
Figure 1: Photographical pictures of different developmental stages of *A. fumigatus* under the microscope. Pictures kindly placed at the disposal by Carla Seegers, Dep. of Clinical Biochemistry (MHH), 5x magnification.

1.4 *Aspergillus* the pathogen

1.4.1 Significance and epidemiology

According to estimations of the Centre of Disease Control and Prevention USA, over five million people are affected by *Aspergillus* infections worldwide (8). Table 1 gives an idea of the global impact.

Aspergillus infection takes place by airborne lung infection (figure 2). It was estimated that human individuals inhale up to 200 *A. fumigatus* spores per day, but their ability to germinate in the human organism and to create an infection depends on the immune status of the host (9). In a healthy immune system, *A. fumigatus* will most likely not evoke any symptoms or physiological alterations. It can even be detected in the sputum of a huge number of immunocompetent healthy beings. Instead, being an opportunistic pathogen, *A. fumigatus* infection is related to a compromised immune system.



In the past having been a rather uncared-for organism in human medicine, nowadays the exploration and characterisation of the *A. fumigatus* pathogen is becoming a more and more crucial concern. Due to growing long-term treatment experience as well as innovations in technology and in treatment possibilities, we are seeing a growing number of immunosuppressed and immuno-debilitated patients. From the infectiological-epidemiological point of view, this development results in a continuously growing number of cases of immunosuppression as well as in a continuously improving long-term survival of the immuno-debilitated individual. These individuals represent the vulnerable population that is predisposed to develop an aspergillosis as a late-onset infection. To give an example, the number of solid organ and bone marrow transplantations is increasing and, hence, we face a raising number of patients under the following live long immunocompromising treatment.

Once established, the *A. fumigatus* infection most likely worsens the prognosis of the underlying disease. For example, manifestation of an *A. fumigatus* infection in the condition of thrombocytopenia increases lethality up to 90%.

Incubation time is extremely variable and depends on the host's immunocompetence. In case of invasive aspergillosis, a lethal outcome can even take place within a few weeks after infection (1).

	Main risk factor	Global burden	Source
Allergic bronchopulmonary aspergillosis (ABPA)	• Allergic asthma • Cystic fibrosis	4.8 million people	Denning et al., 2013 (8)
Chronic pulmonary aspergillosis (CPA)	• ABPA • Tuberculosis • Sarcoidosis • COPD	<u>CPA as a complication:</u> - 400,000 people with ABPA - 1.2 million people with TBC - 70,000 people with sarcoidosis	Denning et al., 2013 (8)
Invasive aspergillosis (IA)	• Immunosuppression • Immunodeficiency	1-2 per 100,000 people with risk factors <u>one-year survival of infected patient:</u> - 59% among solid organ transplant recipients - 25% among stem cell transplant recipients	Estimations of the Centre of Disease Control and Prevention USA

Table 1: The main pathogenetic aspergillosis manifestations in humans in the world, risk factors and global burden.

1.4.2 Virulence factors

The virulence of *A. fumigatus* is associated to its ability to grow in unfriendly environment and to adapt to stress conditions, like the pH or the temperature (see 1.3).

In the host, a pathogenic advantage is the species' strong adaptability to environmental conditions which can be explained by its low requirements in terms of growth conditions and for its capability of genetic adaptiveness. Respective nutritional requirements, *A. fumigatus* can adapt its gene expression and metabolise a variety of nutrients (10). McDonagh et al. found out that certain *A. fumigatus* subtelomeric gene clusters are capable to respond to host-imposed stress, in the shape of iron requirement limitation, alkaline stress or nutrient deprivation, by adapted gene expression in early infection stage. Furthermore, a wide range of secondary metabolites, both candidate or confirmed to contribute to the pathogenicity, is under current investigation, like toxins (for instance, gliotoxin), ROS detoxifying enzymes or the methyltransferase LaeA (11,12).

1.4.3 Risk factors

Foremost neutropenic conditions represent an essential health issue as they debilitate the innate immune system and, thereupon, control of infections is not guaranteed. So not only explicit immunosuppression but any condition or medical treatment debilitating the immune system predisposes for the *A. fumigatus* infection.

Patients that suffer from an HIV infection, from AIDS or from genetically determined immunodeficiencies, as well as patients with hematologic-oncologic or autoimmune underlying diseases, with granulomatosis, thrombocytopenia or leukopenia are vulnerable for an *A. fumigatus* infection as well as patients under cytostatic, radiologic, monoclonal antibody treatment (for example TNF α inhibitors) or long-term corticosteroid treatment (1). Further predisposing conditions are a current or cured tuberculosis infection, above all pulmonal cavitary tuberculosis, diabetes mellitus, chronic alcoholism, malnutrition, advanced age, chronic obstructive pulmonary lung disease, prior pneumothorax, treated lung cancer and non-tuberculous mycobacterial infections (13). In patients with chronic pulmonary diseases with bronchiectasis, emphysema or tuberculous cavernae, *A. fumigatus* shows an affinity to colonise these preformed ventilated caves, even in immunocompetent individuals. Peculiar risk factors for allergic manifestations are the hyper-IgE syndrome or certain HLA-restriction types (14).

There is a frequent *A. fumigatus* colonisation of the paranasal sinuses in people with chronic sinusitis. It can be a source of endogenous infection in a secondary neutropenic status and gives reason for chronic sinusitis to be a further risk factor (15).

1.4.4 Pathophysiology of *A. fumigatus* infection

Transmission of *A. fumigatus* infection is air-borne. The infection takes place by inhalation of its spores which attain the upper airways and, eventually, they reach lower airways and lung tissue. Their small diameter of only 2 – 3 μm is in favour of a quick passage to the small and even outer alveoli, the terminal respiratory units of the lungs, as the *Bronchioli terminales* and *Bronchioli respiratorii* have a diameter between 400 and 600 μm (16).

In a healthy individual, the spores reach the bronchial mucosa where they are eliminated by the mucociliary clearance or, if not so, encounter the cells of the monocyte-macrophage-system that are to be found in the alveoli and ensure a quick elimination (3). In the process of spores' elimination, the innate and the adaptive immune system work together: the innate immune system provides the macrophages which are responsible for phagocytosis and recruitment of neutrophils via a pro-inflammatory response, e.g. TNF α . Neutrophils are responsible for the production of reactive oxygen species that damage different cell components of the conidia and, moreover, neutrophil aggregates inhibit germination which would be the next step in fungal growth. The adaptive immune system acts by antigen presenting cells (APCs) that induce CD4 $^{+}$ cells to differentiate into *A. fumigatus* specific Th1 cells. By INF α production, the latter stimulate macrophages for a more efficient antifungal defence.

The immune system's eliminatory capacity might be exceeded in case of a diminished function and/or a massive exposition to pathogens so that conidia succeed in developing to hyphae (mycelium). In this case the next level of immune defence in terms of granulocytes and T-cells is responsible for hyphae elimination (1).

Any dysfunction in the defending mechanisms described above advantages the invasive fungal infection (7). Thus, individuals at risk are mainly patients that suffer from diseases that disturb the efficacy of the immune system, like hemato-oncologic or immunocompromising conditions, for example therapeutic immunocompromising treatment after organ or bone marrow transplantation. An essential problem is neutropenia. In case of a diminished number of neutrophils the innate immune system is debilitated and the control of infections not guaranteed (3).

1.4.5 Clinical-pathological presentation

Pulmonary aspergillosis can manifest as acute or chronic disease. Further classifications refer to possible local or systemic affection.

In an atopic or predisposed individual, the spores can provoke a type I (IgE-mediated) or type III (immune complex-mediated) *Aspergillus*-related allergic reaction, a so called **allergic bronchopulmonary aspergillosis (ABPA)**. It is known as an allergic respiratory complication in cystic fibrosis or asthma patients as both entities present changes in the airway mucosa which compromise effective clearance from *A. fumigatus* conidia. Genetic predisposition, like some HLA II polymorphisms, is assumed to play a role in development of *A. fumigatus* sensitisation and ABPA. In these individuals, superficial conidia antigens stimulate an inflammatory response, which however is not associated to an effective innate Th1 immune response as, in a healthy system, it should be dominated by macrophages and neutrophils. Instead, the following immune process is pathologically deviated towards a Th2 response which is not able to eliminate the *A. fumigatus* conidia but provokes eosinophils and B cell activation and, hence, IgE liberation. The latter stimulates mast cells and triggers basophil activation and degranulation. Following symptoms are hyper-responsiveness with recurrent pulmonary exacerbations with auscultation crackles and digital clubbing, airway tissue inflammation and stimulation of mucus production which will, in the long run, lead to further atelectasis and bronchiectasis. ABPA typically goes along with bronchiolitis and eosinophilic pneumonia (14).

The **chronic pulmonary aspergillosis (CPA)** can be subdivided into simple aspergilloma, chronic cavitary pulmonary aspergillosis, chronic fibrotic pulmonary aspergillosis, *Aspergillus* nodule and subacute-invasive aspergillosis (13).

If a single “fungus ball” in a pulmonary cavity can be found it is a matter of **simple pulmonary aspergilloma**. Aspergillomas consist of an entangled mycelium and calcium oxalate crystals (3,17). The aspergilloma manifestation is limited to the organ but can evolve to an invasive aspergillosis in case of immunosuppressive conditions, like neutropenia (1). The patients are not immunocompromised and may present few or may not at all present pulmonary symptoms and keep a stable course of disease without progression, though, there is laboratory-confirmed evidence of *A. fumigatus* over at least three months.

The **chronic cavitary pulmonary aspergillosis (CCPA)** shows pulmonary and/or systemic symptoms as well as radiological progress of cavities over at least three months with laboratory-confirmed evidence of the agent. Over the time, cavities can converge and form extensive atelectasis or pulmonary infiltrate. By pleura perforation, the pathogen can reach the pleural cavity. Possibly one lobe can undergo a fibrotic transformation within this entity. In case of a fibrotic reorganisation of two or more lobes, the lung affection is called a **chronic fibrotic pulmonary aspergillosis (CFPA)** and, in the first instance, is the result of a long-time aspergillosis. The final loss of lung tissue causes dyspnea and chronic hypoxia.

Instead, ***Aspergillus* nodules** (> 3 cm) are an unusual way of manifestation and rarely cavitate or infiltrate but may cause tissue necrosis within the nodule centre. Histology is required for a final diagnosis as the differential diagnosis in radiological imaging of pulmonary tuberculoma, *Cryptococcus* nodule and other nodular manifestations is exacting.

In mildly immunocompromised individuals, *A. fumigatus* can cause a **subacute-invasive aspergillosis (SAIA)** which is characterised by a clinical-pathological progress within less than three months. A variegation of radiological manifestation can be found, like nodules, cavities and abscesses. Usually, the SAIA develops in immunocompromised individuals.

The **invasive aspergillosis (IA)** which affects the organism beyond the borders of the lung does not only attack the explicitly immunocompromised individual. Systemic invasion depends on the immune system's ability to prevent *A. fumigatus* from penetration of tissue barriers. Also, for example, recurrent bacterial pneumonia in terms of a superinfection, chronic alcohol abuse or high dose corticosteroids are in favour of this kind of infection (17). Dissemination takes place *per continuitatem* as well as hematogenously. Thanks to a hard cell wall and strongly polarized and, thus, destroying hyphal growth, vessel invasion proves to be a minor problem for *A. fumigatus*. The infection of any organ is possible as well as an *Aspergillus* thrombosis or embolisation or a blood vessel rupture due to platelet activation and the vessel tropism of the hyphae (3). Clinical manifestation can include typical pneumonia symptoms but might lack them in case of an immunocompromised organism. Tissue infarction can produce pain and dyspnea. Extrapulmonary manifestation may appear in shape of fungal sinusitis, cutaneous populous rash, necrotic tracheobronchitis, ophthalmitis or encephalitis. In the central nervous system (CNS), *A. fumigatus* mostly shows up as an abscess. Penetration of the CNS represents a critical situation as the rather lipophobic antifungal drugs have difficulties in overcoming the blood-brain barrier (10).

Individuals at risk are patients in any immunocompromising situation like described in 1.4.3.

1.4.6 Diagnostics

The diagnosis of an aspergillosis turns out to be complex as both clinical presentation and imaging are very unspecific. The adequate therapeutic treatment might be delayed due to the difficulties in defining the diagnosis and because distinguishing the clinical symptoms of an *A. fumigatus* infection from other fungal or bacterial infections is challenging.

Diagnostic methods are rather unspecific as several fungal and bacterial infections as well as lung carcinoma might mimic some aspergillosis features.

Aspergillosis findings change slowly and the diseases's characteristics will be consistent for at least three months in case of a CPA. In immunocompromised patients with SAIA or APA, diagnostic findings change within a shorter lapse of time.

Depending on the aspergillosis sub-entity, **thoracic CT imaging** can show different pulmonary findings. Aspergilloma can be found, mostly upper lobe-located, as ground-glass coloured halo sign which is called air-crescent sign and represents a transient finding. Likewise, one can detect wall thickened cavities, either single-standing or in advanced disease condition coalescing. Atelectasis, pulmonary infiltrate, secondary fibrotic processes of one or more lobes, nodules, empyema or abscesses represent further possible manifestations. Less frequent arterial enlargements or pseudo-aneurisms can be found. In ABPA, mucoid impaction of the bronchi is detectable in ~20% of the cases.

For direct evidence of *A. fumigatus*, **bronchoalveolar lavage (BAL)** fluid specimen, sputum, serum or other fluids might serve for the detection of *Aspergillus galactomannan antigen* using a double-

sandwich enzyme-linked immunoassay in which IgM antibody binds the galactomannan β -(1,5)-galactofuranosyl residues. Alternatively, *Aspergillus* DNA can be detected in a **PCR assay** (18). Due to the underlying immunodepression and the higher fungal burden, in a SAIA or an APA, it is more likely to find specific antigens.

By **lung biopsy**, in a microscopy sample the presence of fungal hyphae or even tissue invasion of hyphae can be proved, the latter in case of SAIA or APA. Hyphae can also be found in sputum or BAL. But the detection of spores or hyphae in the patient's sputum is not a clear evidence for infection as, given the ubiquitous appearance of *A. fumigatus*, falsely positive results must be considered. Less common is a falsely positive BAL result for hyphae in colonised individuals.

Though also people with *Aspergillus* airway colonisation may present increased serological IgG levels, **measuring the immunological response** in shape of circulating *Aspergillus* antibodies is a diagnostic key method as it allows in the most instances the differentiation between colonisation and infection in non-immunocompromised patients with elevated IgG levels in CPA entities and, less studied, elevated IgE levels in asthma, mucoviscidosis and ABPA patients, but the method lacks reliability in case of immunodeficient health situation (19).

In ABPA further methods like measuring basophil activation in a flow cytometer via surface markers, skin test reactivity to antigens or measuring of circulating antibodies are useful methods (14).

Eventually, respiratory fungal and bacterial **culture** of sputum or BAL can help in the differential diagnosis (13).

1.4.7 Antifungal therapy

Fungal cell membrane and, above all, cell wall components differ from mammal cell architecture and are viable drug targets (see figure 3). The antifungal arsenal in clinical use against *Aspergillus spp.* infection comprises amphotericin B and nystatin, two **polyenes** which bind to the fungal plasma membrane protein ergosterol and, thus, disturb the plasma membrane integrity. As a not resorbable agent, nystatin is only of prophylactic or local intestinal use. However, the polyenes show severe side effects, especially nephro- and hepatotoxicity.

Better current therapeutic options are given by the imidazole deriving **triazoles** that include fluconazole, ketoconazole, itraconazole, posaconazole and voriconazole. As outlined in the prior example, the mechanism of action targets the plasma membrane structure as it complexes and inhibits the ergosterol biosynthesis by inhibition of the fungal lanosterol-14- α -demethylase which transforms lanosterol to 4,4-dymethycholesta-8,14,24-trienol (20).

The latest among the clinically active antifungals are the **echinocandins**, like caspofungin, micafungin, ciclofungin or anidulafungin, that constitute an effective therapeutic option. They work by impeding the synthesis of β -(1,3)-glucan, a major cell wall and cell membrane polysaccharide (7). The pharmaceutical options and their targets are listed in table 2.

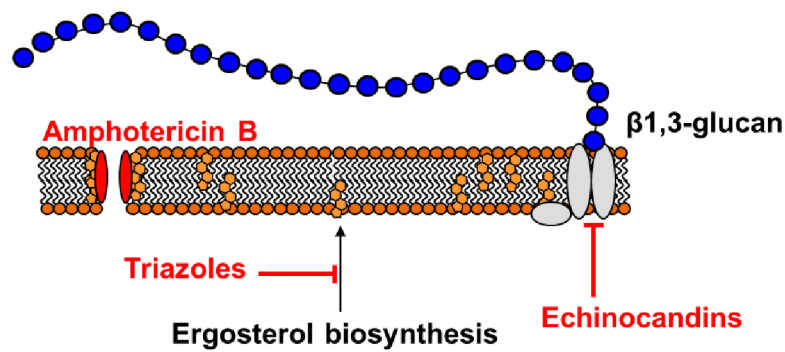


Figure 3: Antifungal drugs target cell membrane components or their synthesis mechanism.

	Target	Considerations
Polyenes 1.) Amphotericin B 2.) Nystatine	cell membrane ergosterol	low selectivity 1.) nephrotoxicity, hepatotoxicity 2.) only local effect
Triazoles	cell membrane ergosterol biosynthesis	many drug interactions (above all CYP3A4 system interactions)
Echinocandins	cell wall and cell membrane β -(1,3)- glucan synthesis	does not penetrate in CNS, should be combined with triazole therapy

Table 2: Current clinical antifungal drugs in *Aspergillus* infection comprise polyenes, triazoles and echinocandins; they hit cell membrane and cell wall targets.

There are further known antifungal substances that act on *Aspergillus spp.* but that are not in clinical use. These comprise nikkomycins and polyoxins, including their synthetic derivatives, that inhibit chitin synthase activity by UDP-N-acetylglucosamine (UDP-GlcNAc) substrate analogy and, this way, affect cell wall integrity. Also comprised are the newly upcoming sordarin derivatives that inhibit protein synthesis by stabilising the ribosome-EL2 complex (21). Another promising approach to find potential antifungal

agents is further research concerning cell membrane synthesis inhibiting pathways, namely phospholipid, sterol and sphingolipid biosynthesis inhibitors. For example, allylamines, like terfenadine or naftifine inhibit selectively the squalene epoxidase, a fungal-specific enzyme. Also, for example, next generation azoles, the modified 1,2,3-azoles, like isavuconazole, ravuconazole and albaconazole specifically target fungal sterol synthesis. But numerous cell plasma membrane synthesis involved molecules can identically or similarly be found in animal cells. So, for example, ceramide synthase inhibitors australifungin and, primarily, fumonisin showed antifungal activity but also toxicity to animal cells. Also, the HMG-CoA-reductase, which is involved in fungal sterol synthesis and can be inhibited by statins, can be found in human cholesterol synthesis. In humans, statins are even the first line drug for the diminution of LDL-cholesterol (20).

So, for the promising adequacy of cell membrane component as drug targets on the one hand but the overlapping of the structures in fungi and animal cells on the other hand, in the near future the scientific community needs to identify reliable, fungus- or species-specific targets.

In case of **CPA** which is a superordinate concept of chronic aspergillosis diseases, detailed treatment depends on the respective entity. Common principle is the first line application of oral triazoles such as itraconazole, posaconazole or voriconazole aiming at the prevention of fibrosis or haemoptysis and at the final healing. Progress in CPA therapy is known to be slow so that duration of treatment is determined for six months with possibly extension to nine months. A lack of therapy progress can be observed consistent with rising azole-resistances so to speak of stable or deteriorating course of disease (22). Next treatment step option represents intravenous application of amphotericin B or echinocandins like caspofungin, micafungin, ciclofungin or anidulafungin. The more aggressive **aspergilloma** or **CCPA** treatment possibility is the local cavity therapy which can be realised by direct cavity injection by amphotericin B, azoles, sodium iodide or nystatin.

More invasive and more radical strategies to limit haemoptysis can be partial lung resection or embolisation.

The **ABPA** is treated with first line treatment of systemic or inhaled corticosteroids in order to prevent inflammation and exacerbation. For the prevention of the side effects of long-term corticosteroid treatment, addition of oral azole antifungal drugs or inhaled amphotericin B can reduce the required corticoid dose. Furthermore, an optional treatment in severe ABPA is immunotherapy with omalizumab, an anti-IgE monoclonal antibody that inhibits mastocytes and basophils from degranulation and, by this means, limits the allergic autoimmunological reaction (14).

In an **invasive aspergillosis**, first line standard therapy consists of voriconazole. In the light of growing resistance rates, voriconazole can be replaced by posaconazole or echinocandins. The latter seems to be more successful and to reduce mortality when applied with voriconazole as combination therapy (23).

1.4.8 Clinical relevance of research

Focus of our research is the identification and characterisation of molecules that are specific for *A. fumigatus* and that might play a role in aspergillosis pathogenicity. These structures can serve as possible pharmacological targets. Molecules that do not exist in mammals are particularly attractive since their inhibition would not interfere with the patient's organism under therapy. Their study might provide information on the pathophysiologic mechanisms so as secondary strategies for prevention can be developed.

1.5 Approach to *A. fumigatus* characterisation

1.5.1 *A. fumigatus* cell wall and cell membrane (24,7,20)

The vital importance of the fungal **cell wall's** integrity can be explained by its various mandatory functions like adhesion, cell-cell-communication or fusion as well as protection against foreign substances, cell shaping and host tissue invasion.

The cell wall of fungi consists of glycoproteins and polysaccharides, among chitin, β -(1,3)-glucan and mannan. The presence and quantity of these and of further cell wall components are determined by the fungal species. In the cell wall, chitins and β -(1,3)-glucan can mainly be found in sites of active cell growth and of spore detaching as they are indispensable for structuring and reorganisation of the wall.

Chitins are β -(1,4)-GlcNAc polymers that crystallise by forming hydrogen-bond. The polymer is synthesised into the cell wall space by the membrane-bound chitin synthase which is encoded by the *A. fumigatus* *chsA* through *chsF* genes and secondarily integrated into the wall.

The predominant cell wall glucan is the **β -(1,3)-glucan** whose synthesis by the membrane-bound glucan synthase complex and integration occurs analogously to chitin. They become decorated by the addition of further glucans. These branched glucans crosslink with other cell wall components. A glucan synthase subunit is assumed to be the target structure of the antifungal group of echinocandins.

Cell wall standing **glycoproteins**, instead, are synthesised and elaborated in the ER and the Golgi apparatus where they are decorated with N- and O-linked polysaccharides. N- and O-linked protein glycosylation is highly conserved among eukaryotes, but specific glyco-decoration patterns distinguish the eukaryote species; so, for example, in *A. fumigatus*, main N-glycosylation components are branched galactomannan structures. In comparison, O-linked components have a simpler structure.

The cell wall and cell membrane glycoproteins enter the secretory pathway mediated by a N-terminal signal. In presence of a specific C-terminal signal, the respective glycoproteins become equipped with a GPI (glycosylphosphatidylinositol) that anchor them to the membrane. GPI-anchored proteins either remain into the plasma membrane or are transferred to the cell wall where they are covalently linked to the β -glucans via a GPI remnant. Many cell wall GPI-anchored glycoproteins are putative enzymes involved in cell wall synthesis and remodelling. Built up by structural proteins on the one hand and by enzymatically active proteins on the other hand, the cell wall is a dynamic formation. So, further refinement of the cell wall composition can take place as adaptation to changes in the environment.

Chitin, β -(1,3)-glucan, cell wall glycoproteins and GPI-anchors are essential components for cell wall and cell membrane integrity and, thus, for fungal viability.

The function of the **cell membrane** includes signal transduction, protection and maintenance of the intracellular milieu and synthesis of cell wall and cell membrane components. The predominant components of the fungal **cell membrane** are glycerophospholipids, sterols and sphingolipids, but also glycoproteins play a functional role.

Glycerophospholipids are a product synthesised in several steps from glycerol-3-phosphate and substituted with groups like serine, glycerol, inositol, mono- and diethanolamine and choline.

Sterols are an amphiphilic major lipid component of the plasma membrane and synthesised from AcetylCoA. Fungal cells share various steps with animal cells in a common sterol building path, but the latter synthesise cholesterol, whereas the fungal counterpart major membrane sterol is ergosterol, a provitamin of vitamin D₂, synthesised from mevalonate across squalene in several steps. Different antifungal drugs in clinical use interfere with the ergosterol synthesis (see 1.4.7).

Sphingolipids are synthesised from palmitoylCoA and serine across the formation of a ceramide which becomes a glycosphingolipid (GSL) by glycosylation with glucose, galactose, galactofuranose, mannose or glucosamine, in case of glycoinositolphosphoceramides (GIPCs), after substitution with phosphatidylinositol (PI).

1.5.2 Glycoinositolphosphoceramides (7,25,26)

Glycosphingolipids (GSL) are an essential component of many eukaryotic cell membranes. Together with ceramides and sphingomyelin, GSL belong to the group of **sphingolipids**. The basic molecule of a sphingolipid is a phytoceramide synthesised from phytosphingosine. The sphingolipids' amphiphilic character predisposes them for a localisation in the cell membrane where, among others, they are responsible for membrane fluidity (26).

A sphingosine bound to a saturated fatty acid chain of 18 to 26 carbon atoms, and a carbohydrate rest, glycosidically bound to the 1-hydrogen group, form the basic structure of **GSL**. The group of GSL contains globosides, gangliosides and cerebroside.

In yeast and fungi, the GSLs can be subdivided into neutral glycosylceramides (β -glucosyl- and β -galactosylceramide, also called gluco- and galactocerebroside), neutral saturated ceramides with a complex moiety and the acidic **glycoinositolphosphoceramides** (GIPC). The GIPC represent a distinct class of complex glycosphingolipids. As indicated by their name, the lipid part of GIPC is formed by inositolphosphoceramide (IPC).

The **IPC core structure** is synthesised by the IPC-synthase that transfers myo-inositol-1-phosphate from phosphatidylinositol to the phytosphingosine-deriving phytoceramide bound to a saturated long chain fatty acid consisting of 18 up to 26 carbons (26).

The IPC-synthase is not present in animal cells, in which the analogous sphingomyelin synthase transfers phosphocholine to ceramide to form sphingomyelin (27).

The expression of IPC is essential for viability of fungi including *A. fumigatus*. Deletion of the IPC-synthase encoding gene *AUR1* caused accumulation of ceramide and led to apoptosis of the cells (28). In contrast, elongation of IPC with mono- or oligosaccharides is not essential for viability (29,30).

The **GIPC subcategories** are characterised by the type of sugar bound to the IPC inositol ring as well as the linkage. In this way, three types of GIPCs were identified in fungi as Man- α -(1,2)-IPCs, Man- α -(1,6)-IPC and GlcN- α -(1,2)-IPCs.

In fungi, IPC is typically substituted by a mannose linked in α -(1,2) bond to the inositol to form MIPC. Additional monosaccharides (mannose and galactofuranose) may elongate MIPC and form a family of acidic GIPC. In a few pathogenic fungi (*A. fumigatus*, *S. schenkii*, *T. viride*, *Acremonium spp.*) another type of GIPC is common as a glucosamine can be linked in α -(1,2) to IPC and elongated by one or two monosaccharides. The positive charge on the amino group of the glucosamine and the negative charge of the phosphate give a zwitterionic character to the latter GIPC (25,31).

1.5.3 Our focus: the zwitterionic glycolipid Af-3c

The prevailing *A. fumigatus* GIPC is the zwitterionic glycolipid **Af-3c** which is located in the fungal plasma membrane. To date, this unusual zwitterionic glycolipid made of Man- α -(1,3)-Man- α -(1,6)-GlcN- α -(1,2)-IPC has only been described in *Aspergillus fumigatus*.

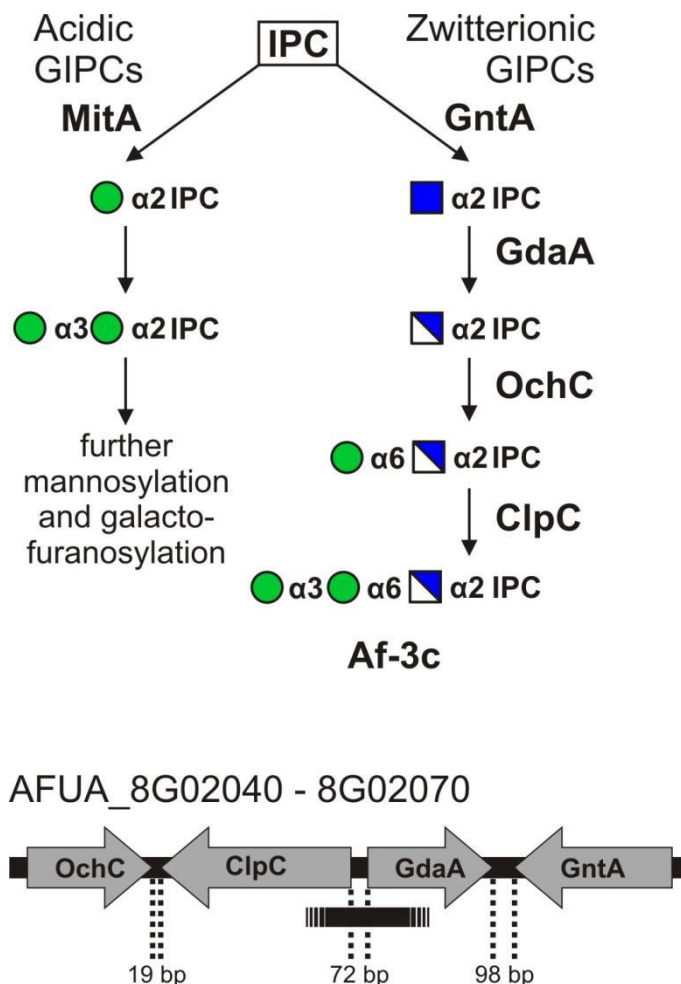


Figure 4: *A. fumigatus* synthesises both acidic and zwitterionic GIPC. The gene cluster necessary for Af-3c production comprises the genes AFUA_8G02040 to AFUA_8G02070.

The Af-3c molecule is synthesised by the gene cluster containing the genes AFUA_8G02040 to AFUA_8G02070 found on chromosome 8 of the *A. fumigatus* genome. This gene cluster encodes four different enzymes: the **N-acetylglucosamine transferase (GntA)** adds an N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to IPC, the **de-N-acetylase (GdaA)** removes then the N-acetyl group of GlcNAc to generate GlcN- α -(1,2)-IPC. Finally, the **α -(1,6)-mannosyltransferase (OchC)** and **α -(1,3)-mannosyltransferase (ClpC)** extend this core (figure 4). The

GlcNAc transferase GntA has already been characterised and its catalytic site was found out to be localised to the lumen of the Golgi apparatus, where synthesis of Af-3c most likely takes place. Deletion of *GntA* in *A. fumigatus* resulted in absence of zwitterionic GIPC proving the enzyme function and establishing that Af-3c is not essential for viability *in vitro* (29).

Engel and colleagues also introduced the genes encoding GntA, GdaA, OchC and ClpC in the yeast strain *Saccharomyces cerevisiae* (*S. cerevisiae*) and generated a glycolipid product having the molecular mass of Af-3c (unpublished result). However, this strategy provided low amount of product, which limited further analyses. *In vitro* transport assays using yeast microsomes have demonstrated that import of UDP-GlcNAc in organelles of the secretory pathway is low in yeast (32). A low import of UDP-GlcNAc in the Golgi apparatus likely limits production of Af-3c. Interestingly, the gene AFUA_8G02090 located downstream of the above-described gene cluster is predicted to encode a nucleotide sugar transporter (NST). It is thus possible that this gene encodes a UDP-GlcNAc transporter that is involved in Af-3c biosynthesis.

1.5.4 Importance of zwitterionic GIPC

The clustering of Af-3c biosynthetic genes suggests that the zwitterionic GIPC provides a benefit to the fungus under specific environmental conditions. However, the function of the zwitterionic GIPC Af-3c in *A. fumigatus* is currently unknown. As mentioned above, a very few phytopathogenic fungi have been shown to produce similar GIPC containing a GlcN- α -(1,2)-IPC core (31). Analysis of the phylogenetic distribution of the Af-3c gene cluster confirms the rarity of such molecules ((33), unpublished results).

Aoki et al. (25) took a closer look at the pathogenicity of zwitterionic glycolipids linked via a GlcN- α -(1,2)-IPC linkage and extracted it from the filamentous fungus *Acremonium spp.* In *Acremonium*, the GlcN- α -(1,2)-IPC core is elongated by an α -(1,6)-linked mannose. They found out that these glycolipids induced cell death in rice cells.

GPIs can also be found in protozoans where they constitute a major portion of the plasma membrane as well as they circulate as soluble GPI form. Fungal zwitterionic GIPC resemble free GPI synthesised by protozoan parasites such as *Leishmania*, *Trypanosoma* or *Plasmodium* (34). In the latter parasite, free GPI are pro-inflammatory endotoxins that may contribute to pathogenesis of malaria. Anti-free-GPI-IgGs are found in malaria patients and may protect against mild and severe malaria (27).

1.6 Aims

Aim of this work is to analyse the biosynthesis of the *A. fumigatus* zwitterionic glycolipid called Af-3c and its function.

Research around *A. fumigatus* pathogenicity for humans and around its cure is scarce. The major reason therefore seems to be that this fungus only recently in the history of medicine developed from orphan pathogen, that in the past caused exotic infections in rare cases, to a seriously harmful and lethal organism nowadays.

During the last decades, therapy strategies from different medical areas that, secondarily, entail immunodeficiency as an aversive effect and, thus a potential risk situation for patients to become a victim of opportunistic pathogens like *A. fumigatus*, developed rapidly. Unfortunately, counterpart achievements in the field of *A. fumigatus* antifungals did not develop at the same speed, even though the group of immunocompromised patients is by itself at a higher risk of a lethal outcome in case of infection.

All the more, modern medicine requires new and more efficient strategies than the current possibilities at our disposition to confront opportunistic *A. fumigatus* infections.

By genetic and molecular research on the *A. fumigatus* organism and metabolism, one might take a step towards the identification of possible target structures that do not have a mammalian counterpart and which, thus, can serve as a base for future specific antifungal drug therapy research.

As already shown not only for infections by *Aspergillus spp.* but in general antifungal treatment, for their molecular structure that differs from mammal cell structure, fungal cell membrane and cell wall components serve as efficient drug targets. The current treatment strategies in clinical use in fact act on either cell wall or cell membrane components.

For this reason, the analysis of the zwitterionic glycolipid Af-3c as a specific *A. fumigatus* plasma membrane component is promising, particularly because this molecule is presumably involved in the *A. fumigatus* pathogenicity mechanism. In this work, we want to characterise a putative transporter involved in Af-3c biosynthesis and estimate the importance of this glycolipid for virulence.

To demonstrate the function of the putative UDP-GlcNAc transporter (UGT) encoded by AFUA_8G02090, we wish to express this protein in yeast cells. Successful recombinant expression of UGT and its presence in isolated Golgi-rich fractions will be demonstrated using Western blot. Subsequently, *in vitro* transport assay using radioactively labelled substrates and the isolated Golgi-rich fractions will prove its function as UDP-GlcNAc transporter.

We further wish to start characterising Af-3c. Wild type reference is the *A. fumigatus* strain D141, which is a hospital isolate, and its derivative AfS35. The Δ gntA strain serves as Af-3c deficient strain, whereas the Δ MitA strain is modified to use IPCs exclusively for Af-3c production. As control, the *gntA* gene was also re-inserted in the Δ gntA strain and the resulting strain called Δ gntA-reintro. Glycolipid can be extracted from spores and hyphae of the strains mentioned above and analysed using thin layer chromatography and mass spectrometry to confirm and assess the level of expression of Af-3c in the different strains, according to their respective genetic predisposition.

An approach, established in this work, to assess the importance of Af-3c in the fungus virulence will be the infection of A549 carcinoma cells with *A. fumigatus* spores. Spores are the form of *A. fumigatus* that *in vivo* first gets in touch with the human lung epithelia and, hence, this entity starts the interaction with the human host cells. Cell damage created by the fungus reflects its virulence and can be used as a method for the selection of strains to investigate further.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Buffer and media

Buffer and media	
BUFFER	
PBS	10 mM sodium phosphates 150 mM NaCl at pH 7.4
PBST	PBS 0.5% Tween
TAE	40 mM Tris 1 mM EDTA 0.1% C ₂ H ₄ O ₂ at pH 8.5
TBE	100 mM Tris-HCl, pH 8.0 100 mM borate 2.5 mM EDTA
TE	10 mM Tris-HCl 1 mM EDTA at pH 8.0
DNA loading buffer	30% glycerol 60 mM EDTA 1x TBE at pH 8.0
T4 Ligase reaction buffer	50 mM Tris-HCl 10 mM MgCl ₂ 1 mM ATP 10 mM DTT at pH 7.5
NEB Cutsmart buffer	50 mM K-C ₂ H ₄ O ₂ 20 mM Tris-C ₂ H ₄ O ₂ 10 mM Mg(C ₂ H ₃ O ₂) ₂ 100 µg/mL BSA at pH 7.9 (25°C)
NEBuffer 1.1	10 mM Bis-Tris-Propane-HCl 10 mM MgCl ₂ 100 µg/mL BSA at pH 7 (25°C)

NEBuffer 2.1	50 mM NaCl 10 mM Tris-HCl 10 mM MgCl ₂ 100 µg/mL BSA at pH 7.9 (25°C)
NEBuffer 3.1	100 mM NaCl 50 mM Tris-HCl 10 mM MgCl ₂ 100 µg/mL BSA at pH 7.9 (25°C)
Zymolase buffer	50 mM KPO ₄ buffer 1.4 M Sorbitol 10 mM NaN ₃ 40 mM β-mercaptoethanol at pH 7.5
Lysis buffer Golgi prep	10 mM HEPES/Tris 0.8 M Sorbitol 1 mM EDTA at pH 7.4
2x Laemmli, 8 M Urea	200 mM Tris-HCl 30% (v/v) glycerol 3% (w/v) SDS 8 M Urea at pH 6.8
SDS running buffer	50 mM Tris 350 mM glycine 0.1% SDS
Blotting buffer	48 mM Tris 39 mM glycine
Blocking buffer	2% (w/v) milk in PBS
Assay buffer	10 mM Tris-HCl 2 mM MgCl ₂ 0.8 M sorbitol at pH 7.0
Breaking buffer	50 mM NaPO ₄ 1 mM EDTA 5% glycerol 1 mM PMSF at pH 7.4
HPTLC solutions:	
Solvent	CHCl ₃ :MeOH:H ₂ O(0.038% (w/v) CaCl ₂) [50:47:14]
Carbohydrate staining solution	MeOH:H ₂ O (0.5% (w/v) orcinol; 5.5% (v/v) H ₂ SO ₄) [8:3]

COMPOSITION OF BACTERIA MEDIA

LB medium	10 g/l tryptone 5 g/l yeast extract 10 g/l NaCl
LB agar with ampicillin	LB medium 1.5% (w/v) Bacto agar at pH 7.5 (NaOH) 100 µg/mL ampicillin

COMPOSITION OF YEAST MEDIA

SC medium	1.7 g/l Difco yeast nitrogen base w/o AA 5 g/l (NH ₄) ₂ SO ₄ glucose 2% selective amino acids 1x
SC agar	SC Medium + 2% (w/v) select agar
20x AA Mix	2 g/l leucin and/or 1 g/l histidine and/or 1 g/l methionine and/or 2 g/l uracil

COMPOSITION OF *A. FUMIGATUS* MEDIA

1000x trace elements	4.4 g ZnSO ₄ · 7H ₂ O 2.2 g H ₃ BO ₃ 1 g MnCl ₂ · 4H ₂ O 1 g FeSO ₄ · 7H ₂ O 0.32 g CoCl ₂ · 6H ₂ O 0.32 g CuSO ₄ · 5H ₂ O 0.22 g (NH ₄) ₆ Mo ₇ O ₂₄ · 4H ₂ O 10 g Na ₄ -EDTA at pH 6.5 10 M KOH at ddH ₂ O to 200 mL
50x AspA salts	3.5 M NaNO ₃ 550 mM KH ₂ PO ₄ 350 mM KCl ad pH 5.5 10 M KOH
AMM (Aspergillus minimal medium)	1x AspA salt 1x trace elements 2 mM MgSO ₄ 1% (w/v) glucose pH 6.5
AMM agar	AMM + 2% (w/v) agar
Selective agar/medium	AMM + 30 µg/mL phleomycin or AMM + 200 µg/mL hygromycine or AMM + 0.1 µg/mL pyrithiamine

COMPOSITION OF A549 MEDIA

DMEM F-12 + 10% FCS

inorganic salts, amino acids, vitamins
10% FCS

2.1.2 Primers

Primers

JE83 PCR of AF_90 fragment gcaaGGATCCCTTCGAGTGATGAACATATACAGG (BamHI)

JE102 PCR of AF_90 fragment gcagTCTAGACTACACCACACCTTTGACTTCCCTCTTCTCCGTCACATTGGTT
CCCGTCATACTCTGCCCTGCGAAG (XbaI)

PYesF (for sequencing) GTG CAA TAT CAT ATA GAA GTC A

PYesR (for sequencing) CAG CTA CCA CAT TGG CAT TG

MH178 linker AGCTTcgaATGGGTAAGCCTATCCCTAACCTCTCCTCGGTCTCGATTCTACG
ggtacAGCTTcgaATGGGTAAGCCTATCCCTAACCTCTCCTCGGTCTCGATT
CTACGggtac

MH179 linker cCGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCCATtcgA

FR208 for PCR of Af_90 fragment ctgaGGTACCCTTCGAGTGATGAACATA (KpnI)

FR209 for PCR of Af_90 fragment catgTCTAGACTACACCACACCTTTGAC (XbaI)

FR210 for PCR of SLC35A3 fragment ctgaGGTACCTTGCCAACCTAAAATACG (KpnI)

FR211 for PCR of SLC35A3 fragment catgTCTAGACTCGAGaCTATGCT (XhoI)

2.1.3 Enzymes

Enzymes

Q5 High Fidelity Polymerase

T4 DNA ligase

Restiction emzymes (endonucleases)

- | | |
|--------------------|------------------------|
| - SfiI | - XhoI |
| - BamHI / BamHI-HF | - HindIII / HindIII-HF |
| - XbaI | - ApaI |

- EcoRI	- KpnI / KpnI-HF
- SpeI	- XcmI
- NheI	- BsaI-HF
DNase (RQ 1)	
Reverse transcriptase (Fermentas Revert Aid Premium Superscript)	
Trypsin	
Zymolase	

2.1.4 Tags

Tags	
FLAG	GAC TAC AAA GAC GAT GAC GAC AAG (DYKDDDDK)
V5	CGT ACT GTC GAG GCC CAA CAG TGG ATT TGG TAT TGG CTT TCC (GKPIPNNLLGLDST)

2.1.5 Plasmids

Plasmids	
pYESCup H-FLAG-K (= pYESCup1)	Saccharomyces cerevisiae expression vector for expression of an N-terminally FLAG-tagged protein, modified by Ashikov et al. from the pYES2/NT-C vector (Invitrogen, Thermo Fisher Scientific Inc.) by introducing the promotor cup1 for gene expression. The in-frame cloning sites are KpnI or BamHI. It contains an ampicillin resistance gene.
pESC_URA	<i>S. cerevisiae</i> expression vector, prototrophy for Uracil
pESC_LEU	<i>S. cerevisiae</i> expression vector, prototrophy for Leucine
pYES_URA	<i>S. cerevisiae</i> expression vector, prototrophy for Uracil
➤ _90_FLAG	<i>S. cerevisiae</i> expression vector based on pYESCup H-FLAG-K bearing a FLAG-tagged (BamHI/XbaI) or a V5-tagged (HindIII/ KpnI) UGT gene (AFUA_8G02090)
➤ _90_V5	

2.1.6 Strains

Bacterial and fungal strains	
E. coli XL-1 blue	Genotype: recA1; endA1; gyrA96; thi-1 hsdR17; supE44; relA1; lac [F' proAB lacIq ZΔM15 Tn10 (Tetr)]
BY4741	Wild type yeast strain (EUROSCARF). Genotype: BY4741; MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YEL004w::kanMX4 (low UDP-GlcNAc transport)
K03	Knockout strain, derivate of BY4741 with YEL004W::KanMX4 (deletion of a putative NST, lacks UDP-GlcNAc transport)
AfS35	<i>ΔakuA</i> mutant, derivate of the <i>A. fumigatus</i> clinical isolate D141, deficient in non-homolous end-joining (Krappmann et al.) (35)
ΔgntA	Knockout strain, derivate from AfS35 that lacks the endogenous Af-3c synthesising pathway (Af-3c free control; Engel et al.) (29)
ΔgntA-reintro	Knockout strain, derivate from AfS35 that lacks the endogenous Af-3c synthesising pathway with re-insertion of <i>gntA</i> gene (reconstituted Af-3c synthesis; Engel et al.) (29)
ΔMitA	Knockout strain, derivate from AfS35 that lacks the endogenous acidic GIPC synthesising path (IPCs are metabolised exclusively for Af-3c production; Kotz et al.) (30)

2.2 General molecular biology techniques

2.2.1 cDNA synthesis from mRNA

Complementary DNA (cDNA) synthesis was performed from messenger RNA (mRNA) in order to obtain the pUGT encoding DNA sequence without introns. Freeze-dried mycelium served as RNA providing organic material. After homogenisation by suspension in TRIzol and centrifugation, phases were separated adding chloroform and the RNA extraction continued from the aqueous phase following the Nucleospin RNAII protocol. RNA was bound to a column and desalted using MD buffer. rDNase was applied in order to remove possible genomic DNA. The RNA was washed applying the supplied buffers. Then, genomic DNA was removed by addition of DNase I. First strand cDNA was created by reverse transcription, employing the Fermentas Revert Aid Premium Superscript reverse transcriptase and incubating with random hexamer primers.

2.2.2 Polymerase chain reaction (PCR)

The adapted Q5 High-Fidelity DNA Polymerase protocol was used for DNA amplification.

Components for a 50 μ L reaction	
3 ng	template DNA
0.5 μ M	sense primer
0.5 μ M	antisense primer
10 μ L	5x Q5 Reaction Buffer
200 μ M	dNTPs
	H ₂ O up to 50 μ L
0.5 μ L	Q5 High-Fidelity DNA Polymerase

All reaction components were mixed on ice and quickly transferred to the Biometra T3000 thermocycler. For the amplification we used a denaturation temperature at 98°C. The annealing temperature was adapted to the primer pairs. The PCR cycles were followed by a final elongation of 2 min and a cooling at 4°C.

PCR program		
98°C	30 sec	Denaturation
98°C	10 sec	 25 cycles
62°C	20 sec	
72°C	30 sec	
		DNA copying; 20-30 sec/kb
72°C	120 sec	
4°C	hold	

2.2.3 DNA analysis and purification

0.6%, 0.8%, 0.9% or 1.2% agarose gels were used for electrophoretic separation of PCR product, DNA fragments or plasmids according to their size by band shift in an electric field. The lowest agarose density was chosen for large DNA (approx. 800 – 1,500 bp). Agarose was solved in TAE buffer (40 mM Tris, 1 mM EDTA, 0.1% acetic acid) by boiling and cooled down to approx. 60°C. For visualisation of the DNA by UV light 2.5 μ L ethidium bromide were added to the gel. 5x DNA loading buffer (30% glycerol, 60 mM EDTA, 1x TBE) was added to the samples. Gel electrophoresis was performed at 60 Volt and DNA detected on a UV table. For size evaluation, Quick Load ladders (NEB) were used.

The desired fragment was excised from the gel and purified using the PCR clean-up kit (Macherey-Nagel or QIAGEN) according to manufacturer's information. Elution was usually done in 35 μ L (for PCR, in 50 μ L).

2.2.4 Restriction digest of DNA

Restriction digests were performed using endonucleases from New England Biolabs GmbH (NEB) following the manufacturer's recommendations for buffer and reaction temperature. For analytical digests, 0.5 unit (0.25 µL) of each restriction enzyme were used in a 20 µL reaction. For preparative digests, we applied 1 µg of plasmid with 1 unit (0.5 µL) of each restriction enzyme in a 50 µL reaction. Incubation time of 4 – 5 hours was used for complete digest while 2 – 3 hours were sufficient for test digests. For the latter, we applied enzymes with restriction sites different from the insert ligation sites and preferably cutting within the fragment sequence.

2.2.5 Ligation

0.05 pmol of the digested vector and a 50 times molar excess of the respective insert (2.5 pmol insert) were mixed with 1 Weiss Unit (1 µL) of T4 DNA Ligase (NEB) and 2 µL T4 ligase reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT) were added. We incubated at room temperature for 1 hour and transformed into chemically competent *E. coli* XL-1 blue cells.

2.2.6 Plasmid transformation in bacteria

100 µL of chemically competent *E. coli* XL-1 blue cells were thawed on ice, and mixed with 10 ng of plasmid DNA or 15 µL of the ligation reaction. After 10 min on ice, cells were heated at 42°C for 45 sec and incubated on ice for 2 min before plating. The cell solution was added to LB agar plates (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agar, pH 7.5 using NaOH) containing 100 µg/mL ampicillin. Plates were placed in an incubator at 37°C overnight. A colony was picked up with a toothpick and incubated in LB medium for 12 – 16 hours at 37°C, 250 rpm in 5 mL LB medium with 100 µg/mL ampicillin.

2.2.7 Extraction of transformed DNA from bacteria

For the preparation of plasmid DNA in terms of a mini-prep, we used the Roche kit following the included isolation protocol and eluting the final DNA in 50 µL.

2.2.8 Determination of the DNA concentration and sequencing

DNA concentration was determined photometrically (Ultrospec 7000, dilution factor 80, factor 50). We verified the sequences of the created plasmids by GATC Biotech sequencing.

2.3 Cultivation and transformation of *S. cerevisiae*

2.3.1 General yeast culture

The yeast strain BY4741 having a deletion of the putative NST gene Y00244 with the genotype [MATa; ura3 Δ 0; leu2 Δ 0; his3 Δ 1; met15 Δ 0; YEL004w::kanMX4] was used. This strain presents a low endogenous UDP-GlcNAc transport which turns it into a convenient model for our study of a putative UDP-GlcNAc transporter.

Synthetic medium (SC medium) supplemented 2% glucose and required amino acids (leucine, histidine, methionine and uracil) was used for culture. 2% select agar was added for plates. Yeast cells from a glycerol stock were streaked on a plate and incubated at 30°C for 3 to 7 days. Plates could be stored in the fridge for some weeks.

One colony from a SC agar plate with 2% glucose (per plate 0.034 g yeast nitrogen base, 0.1 g ammonium sulfate, 0.4 g yeast agar, 17 mL H₂O, 2 mL glucose 20%, selective amino acids 1x) was suspended in 10 mL SC medium and incubated at 30°C overnight, 225 rpm. The density was determined by OD₆₀₀ measurement. We employed the Ultrospec 2100pro photometer.

For long term storage, glycerol was mixed to 1 mL culture to obtain 15% glycerol and it was frozen at -80°C.

2.3.2 Transformation in yeast

An overnight liquid culture was diluted in 50 mL SC medium to obtain an OD of 0.4 and grown for 4 hours. Cells were centrifuged at 1,500 x g for 5 min and washed with 40 mL TE buffer. After resuspension in 2 mL 100 mM LiAc/0.5xTE, yeast cells were incubated for 10 min at room temperature. For a better DNA uptake 100 μ g denatured sheared salmon sperm DNA (Invitrogen) were mixed with 1 μ g plasmid DNA and 100 μ L of the yeast suspension. A tube without plasmid DNA was used as negative control. After addition of 700 μ L 100 mM LiAc/40%PEG 3350/1xTE, tubes were incubated at 30°C for 30 min. 88 μ L DMSO were added and cells were heated at 42°C for 7 min. Cells were pelleted, washed once with TE, resuspended in 50 μ L TE and plated on a SC agar plate lacking uracil since the plasmid contains the URA gene. Single colonies were picked after incubation for 3 to 5 days at 30°C.

2.3.3 Induction of UGT expression and preparation of a Golgi-rich fraction

A growing yeast culture was diluted to an OD₆₀₀ of 0.03 in 1 L and cultivated overnight at 30°C, 225 rpm. When the culture reached an OD₆₀₀ of 0.8, protein expression was induced with 0.5 mM CuSO₄ for 3 hours at 30°C, 225 rpm (see figure 5).

Cells were harvested by centrifugation at 1,500 x g for 5 min, 4°C, (Multifuge Heraeus Megafuge 40R centrifuge) and washed with 10 mM NaN₃. The wet cell pellet was weighted (equates to yeast cells in [g]). The cell pellet was resuspended in fresh zymolase buffer (50 mM KPO₄ buffer, pH 7.5; 1.4 M Sorbitol, 10 mM NaN₃, 40 mM β -mercaptoethanol) using 3 mL buffer/g yeast cells. Zymolase 100T (MP Biomedicals from *Arthrobacter luteus*) was added at a concentration of 0.5 mg/mL and the mixture was incubated at 30°C, 75 rpm. After various incubation times (e.g. 15, 20 and 25 min) 10 μ L were diluted into 990 μ L 5%SDS and the OD₈₀₀ was measured. We realised the following steps in the cold room (-20°C).

Spheroplasting percentage could be calculated as follows:

$$100 \times [1 - [OD_{800}(t) / OD_{800}(t=0)]]$$

The reaction was centrifuged at 1,000 x g for 5 min at 4°C when a spheroplasting percentage of 85 – 90% was reached (usually after ~20 min).

The spheroplasts pellet was resuspended in lysis buffer (10 mM HEPES/Tris, pH 7.4, 0.8 M Sorbitol, 1 mM EDTA) with protease inhibitor (Complete Ultra Tablets, EDTA-free (Roche), 1 tablet/50 mL lysis buffer) using 3 mL/g yeast cells. The lysate was filled in a dounce vessel, the centrifugation containers were rinsed with lysis buffer and the dilution added to the vessel. The cell membrane was mechanically lysed in the dounce vessel by ten strokes. To remove intact spheroplasts, the homogenous suspension was centrifuged for 5 min, 1,500 x g, 4°C in the Avanti J-30I Centrifuge and the supernatant was collected. We repeated the lysing step with the pellet and collected the supernatant. The ER-rich fraction was pelleted at 10,000 x g, for 10 min, 4°C, resuspended in lysis buffer using 800 µL/g of yeast cells and stored at -80°C. The supernatant was centrifuged at 100,000 x g for 1 hour, 4°C to pellet the Golgi-rich microsomal fraction (Beckman Coulter Ultracentrifuge).

The pellet was resuspended in lysis buffer using 800 µL/g of yeast cells. 110 µL aliquots of both Golgi-rich and ER-rich fraction were snap freezed in liquid N₂ and stored at -80°C.

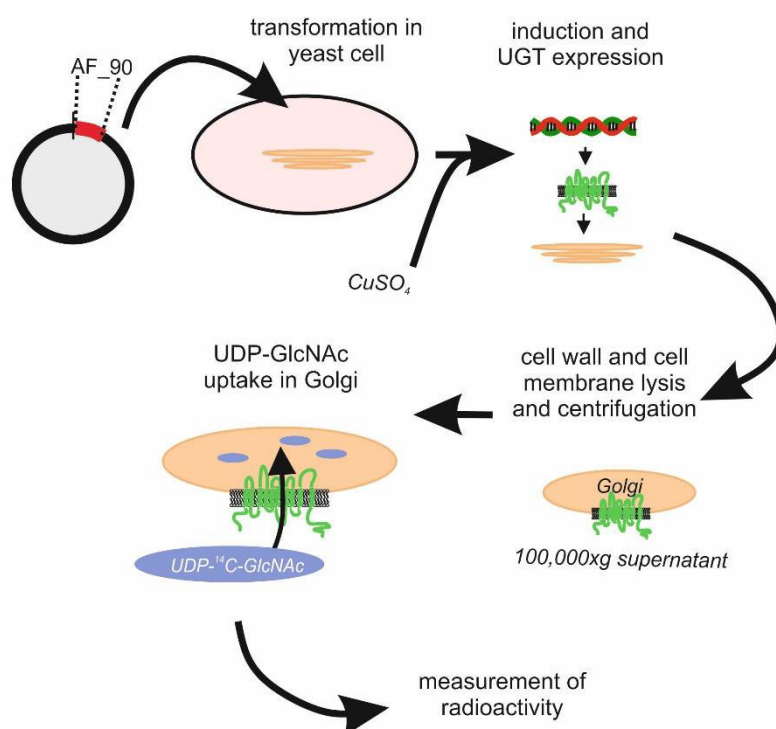


Figure 5: Scheme of induction of protein expression, preparation of Golgi-rich fraction and *in vitro* transport assay.

2.3.4 BCA

This method is based on bicinchoninic acid (BCA) and can be applied for the colorimetric detection and quantification of the total protein in a sample. By the BCA assay we could determine the protein concentration and apply these results to the expression of recombinant protein. Lysates were diluted in NaCl 1:50 or 1:100 and compared to a standard series of known protein concentration. The analysis was carried out applying a microplate reader at 562 nm and the KC4 software. By comparison of the samples to the resulting standard curve, protein concentration could be determined.

2.3.5 Expression of recombinant protein

We followed the adapted Invitrogen pYES transformation protocol for *S. cerevisiae* but using plasmids containing a Cup1 promotor instead of the original Gal1 promotor.

A single yeast colony was cultured overnight in 10 mL SC medium (see above). The yeast culture was diluted to an OD₆₀₀ of 0.4 in 50 mL and protein expression was induced with 0.5 mM CuSO₄. Aliquots were harvested at different time points (e.g. 0, 3, 6, 9 hours) and the OD₆₀₀ was measured. Cells were centrifuged at 1,500 x g, 5 min, 4°C. After washing and pelleting, the cells were resuspended in 500 µL breaking buffer (50 mM NaPO₄, 1 mM EDTA, pH 7.4, 5% glycerol, 1 mM PMSF), centrifuged again at 1,500 x g, 5 min, 4°C. Cell wall was lysed mechanically by vortexing 4 x 30 sec with 1 volume of acid-washed glass beads (560 – 600 µm). After centrifugation in a microcentrifuge at maximum speed the supernatant contained the protein fraction and was kept for BCA assay, SDS-PAGE and Western blot.

2.3.6 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the protein fractions of the samples that we obtained from recombinant protein expression or Golgi preparations according to the protein molecular masses. Negatively charged sodium dodecyl sulfate (synonym: sodium lauryl sulfate) binds to the proteins, unfolds them and serves as an electric neutraliser in that it provides for an adjusted charge-to-mass ratio. The acrylamide portion consists of a 5% acrylamide stacking gel (125 mM Tris-HCl, pH 6.8, 0.1% SDS, 5% polyacrylamide) and a 12% acrylamide running gel (375 mM Tris-HCl, pH 8.8, 0.1% SDS, 12% polyacrylamide). The addition of 0.1% TEMED and 1% ammonium persulfate (APS) started the polymerisation process of both gels which took about 1 hour. Both of the vertical electrophoresis chambers of the SDS-PAGE apparatus were filled with SDS running buffer (50 mM Tris, 350 mM glycine, 0.1% SDS). The lysates were combined with sample buffer 1:1 (2 x Laemmli, 8 M Urea) and incubated at 37°C for 15 min. At least 20 µg of protein per well were loaded. The Precision Plus Protein™ All Blue ladder was used as protein size indication. We ran the gels at 15 to 20 mA per gel (around 70 to 80 Volt) for 1 to 1.5 hours in SDS running buffer whereas the running out of the blue dye from the gel into the buffer indicated the end of electrophoresis.

2.3.7 Western blot

SDS-PAGE gels were washed in blotting buffer (48 mM Tris, 39 mM glycine) and a transversal electric current was applied to the gel in a semi-dry blotting chamber (Biometra) at 2 mA/cm² for 45 min to 1 hour in order to transfer the proteins to a nitrocellulose membrane.

2.3.8 LI-COR spectrometric protein detection

The respective tag could be visualised by indirect fluorescence in a spectrometer. After blotting, the nitrocellulose membrane was blocked overnight in blocking buffer (2% (w/v) milk in PBS (10 mM sodium phosphates, 150 mM NaCl, pH 7.4)).

Then the first antibody with affinity to the respective antigen motif (FLAG tag: DYKDDDDK, or V5 tag: GKIPNPLLGLDST) was diluted 1:5,000 in 2% milk/PBS and incubated for at least 1 hour at room temperature. After 3 washes in PBS for 5 min, the membrane was incubated with an IRDye secondary antibody (see table 3) diluted 1:20,000 in 2% milk/PBS for 1 hour at room temperature. After 3 washes in PBS for 5 min, the nitrocellulose membrane was scanned in the LI-COR Odyssey Infrared Imaging System Scanner in order to visualise the fluorescent signals. The secondary antibody emitted either a red signal at 680 nm or a green one at 800 nm.

Employed LI-COR antibodies

1 st antibody	2 nd antibody
Anti-FLAG M2 rabbit polyclonal antibody directed against DYKDDDDK 0.8 µg/mL	anti-rabbit IR Dye 680 CW (red) 1 mg/mL
Anti-FLAG M5 mouse monoclonal antibody directed against DYKDDDDK 0.8 µg/mL	anti-mouse 680 CW (red) 1 mg/mL
Anti-FLAG M2 mouse polyclonal antibody directed against DYKDDDDK 0.8 µg/mL	anti-mouse 680 CW (red) 1 mg/mL
Anti-V5 mouse monoclonal antibody directed against GKIPNPLLGLDST 0.5 µg/mL	anti-mouse IR Dye 800 CW (green) 1 mg/mL

Table 3: Employed LI-COR antibodies.

2.3.9 *In vitro* transport assay

Transport activity was measured in a radiolabeled nucleotide sugar transport assay. The test was performed as described in the works of Ashikov et al., 2005 (32). 50 μL of a 2 μM UDP-[6- ^3H]-Glc solution (with 0.37 kBq/ μL) or 2 μM UDP-[^{14}C]-GlcNAc solution (with 0.037 kBq/ μL) in assay buffer (10 mM Tris-HCl, pH 7.0, 0.8 M sorbitol, 2 mM MgCl_2) were added to 50 μL Golgi-rich and ER-rich fraction (equivalent to 75 – 100 μg of protein) and incubated for 30 sec at 30°C. Reactions were stopped by dilution with 1 mL of ice-cold assay buffer containing 1 μM respective cold nucleotide sugar. The separation of vesicles and nucleotide sugars that were not incorporated in the compartment was achieved by filtration through nitrocellulose filter (MFTM membrane filters Millipore, Bedford, MA). These steps are described in figure 6. Then, filters were washed three times with 2 mL of ice-cold assay buffer containing the corresponding cold nucleotide sugar at a concentration of 1 μM , in order to prevent export of radioactive nucleotide sugar. Radioactivity associated with the vesicular fraction was measured in a scintillation counter after addition of 3 mL of scintillation liquid. Counts per minute (cpm) that describe the exact quantity of decaying radioactive atoms in one minute were measured and could be converted in disintegration per minute (dpm) which describes the number of decayed atoms based on the efficiency rating of the oscillation counter for the respective isotope:

$$\text{dpm} = \text{cpm} / \text{efficiency}$$

Protein transport in [pmol/mg/min] could be calculated related to the specific activity of the nucleotide sugar reference which was calculated based on reference dpm and protein concentration.

Golgi vesicles from yeast cells transformed with an empty vector were used to measure endogenous transport. The endogenous transport in *S. cerevisiae* of UDP-Glc was used to control the quality of vesicles. In each experiment, nucleotide sugars were assayed in duplicate with the same vesicle preparation.

In vesicle preparations that contain intact membranes, isotope uptake correlates with the transport activity.

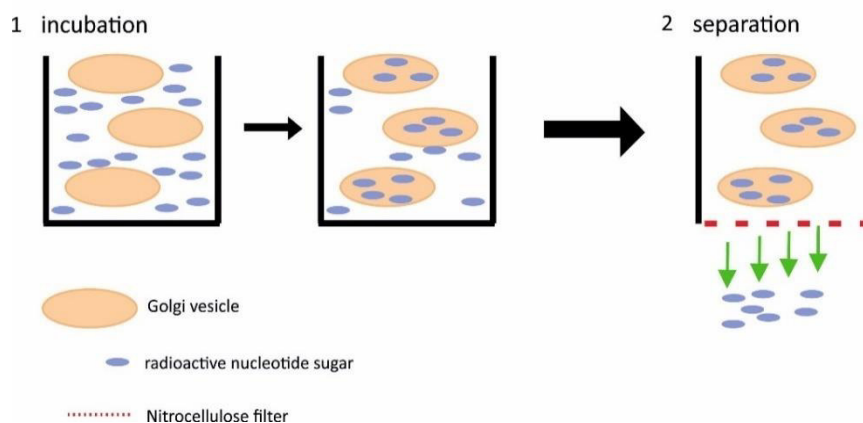


Figure 6: *In vitro* transport assay: vesicles were incubated with radiolabeled sugar that was uptaken into the vesicles by the respective transporter. After washing, the nucleotide sugars that were not incorporated were separated by filtration through nitrocellulose membrane.

2.4 Experiments with *Aspergillus fumigatus*

2.4.1 Culturing *A. fumigatus* spores

A. fumigatus strains:

- AfS35 (wildtype): a derivative from the clinical isolate D141 but deficient in non-homologous end-joining (36)
- Δ gntA: this knockout lacks the Af-3c synthesising pathway and serves as Af-3c free control (29)
- Δ MitA: knockout that uses IPCs exclusively for Af-3c production as the endogenous GIPC synthesising path is knocked out (30)
- Δ gntA-reintro: reintroduced *gntA* gene in Δ gntA restored Af-3c synthesis (29).

Cultivation of the *A. fumigatus* wild-type D141 strain, Δ MitA and Δ gntA-reintro mutants (29,31) was performed in *Aspergillus* minimal medium (AMM). AMM consists of 1% (w/v) glucose, 70 mM NaNO₃, 11.2 mM KH₂PO₄, 7 mM KCl, 2 mM MgSO₄ and trace elements (pH 6.5).

A 1,000x concentrated trace element solution contained 4.4 g ZnSO₄ · 7H₂O, 2.2 g H₃BO₃, 1 g MnCl₂ · 4H₂O, 1 g FeSO₄ · 7H₂O, 0.32 g CoCl₂ · 6H₂O, 0.32 g CuSO₄ · 5H₂O, 0.22 g (NH₄)₆Mo₇O₂₄ · 4H₂O, 10 g Na₄-EDTA, at pH 6.5, 10 M KOH and double-distilled water (ddH₂O) to 200 mL. For solid media, we added 15 g agar/liter AMM. 50 mL AMM agar was poured in vented 250 mL tissue culture flask (Greiner Cellstar 75 cm², 250 mL Flask PS Red filter screw cap Sterile N° 658175), 50 mL agar per flask.

Phleomycin, pyrithiamine or hygromycin were used at concentration of 30 µg/mL, 0.1 µg/mL or 200 µg/mL respectively and served as selection marker for the *A. fumigatus* strains (see table 4).

Antibiotics stock	Final concentration
Phleomycin 30 mg/mL (1000x)	30 µg/mL
Hygromycin 100 mg/mL (500x)	200 µg/mL
Pyrithiamine 300 µg/mL (3000x)	0.1 µg/mL

Table 4: Employed antibiotic impregnation for strain mutant selection.

To collect fresh spores, around 50,000 spores from a glycerol stock were wetted in 1 x PBS and distributed on an AMM agar flask using glass beads (1.5 – 2 mm) and incubated at 37°C, 100% humidity, for 5 days. Spores that are visible as a green-gray layer were dislodged in 10 to 15 mL PBS + 0.05% Tween 20 by shaking with glass beads. Spore suspension was collected and used immediately or stored at 4°C. Long term storage is possible up to years (750 µL spore solution plus 250 µL glycerol 80% (final concentration: 20% glycerol) at -80°C).

Spores were counted in a Neubauer chamber. The average number of 5 x 1 mm² squares was determined and the following formula applied:

$$[\text{number of spores per } 1 \text{ mm}^2 \text{ square}] \times \text{dilution factor} \times 10^4 = \text{spores /mL}$$

Alternatively, the spore concentration was deduced from the OD₄₅₀ of diluted spore suspension. This method was developed by A. Krüger (AG Routier) who defined the linearity between the spore concentration and OD at 450 nm using suspensions with known spore concentration (unpublished results). The spore concentration in spores/mL can be calculated using a slope of 2.174×10^7 and intercept of -8.52×10^4 .

2.4.2 Culturing *A. fumigatus* mycel

To grow *A. fumigatus* mycel, 10^7 spores/mL were inoculated in 100 – 200 mL of AMM and incubated at 37°C, 100% humidity, 300 rpm, for 2 days. Then, the mycelium could be harvested by filtration through a Miracloth filter in a glass funnel after washing 2 x with bidest water.

2.4.3 Clone selection

With the aim to select reference mutants that allowed the analysis of Af-3c expression profiles, genomic resistance properties that served as selection markers allowed the selection of non-contaminated clones. Available clones were screened by plating on antibiotic impregnated selective agars.

2.4.4 Glycolipid extraction

In order to analyse the respective glycolipid profiles, glycolipids were extracted from the *A. fumigatus* spores as well as from mycelium.

To isolate the glycolipid fraction from *A. fumigatus* spores, a spore pellet of 200 – 500 mg in approx. 500 µL was filled up to 600 µL with TE buffer and homogenised with 0.5 mm glass beads for 3 x 20 sec, at 6,800 rpm with 60 sec breaks between each pulse sequence (Precellys 24 homogeniser). After centrifugation in a bench centrifuge for 5 min at maximal speed, the supernatant was collected in a falcon. The pellet was re-extracted in 500 µL MeOH as described above and the supernatant added to the first one. CHCl₃ and MeOH were then added to obtain a final solvent ratio CHCl₃/MeOH/H₂O of 10:10:3 (v/v/v) and disrupted by sonification in a water-ice-bath with 3 x 15 pulses, 50% amplitude. The homogenate was centrifuged for 10 min, 4,500 x g. The supernatant was kept and the pellet re-extracted as described above, first with 2.5 mL of CHCl₃/MeOH/H₂O (10:10:3), then by adding 2.5 mL CHCl₃/MeOH 1:1. The pooled supernatants were centrifuged for 10 min, 4,500 x g and the supernatant was dried under the N₂ stream.

Glycerolipids contained in the extracted fraction, were removed by saponification. The dried glycolipids were re-suspended in 1 volume of 50% propanol-1 in a sonification bath. One volume Ammonia 30% was added and the suspension was incubated for 5 hours at 50°C and dried under the N₂ stream.

The dried glycolipids were resuspended in 1.5 mL butanol saturated with H₂O. Then, 750 µL of H₂O were added and the suspension was vortexed, the phases were separated by centrifugation for 5 min, 300 x g and the butanol phase (upper phase) was transferred to a clean falcon.

The H₂O phase was re-extracted twice. The butanol phases containing the glycolipids were then washed three times with H₂O saturated with butanol and dried under the N₂ stream.

For the re-N-acetylation, the dried glycolipids were resuspended in 2 mL $\text{CHCl}_3/\text{MeOH}$ 1:1, placed in an ultrasonification bath for a few minutes and 10 mg of NaHCO_3 were added. After addition of 80 μL of acetic anhydride, the glycolipids were mixed for 15 min at 500 rpm. This step was repeated four times. 1 mL of both CHCl_3 and 350 μL of H_2O were added to obtain the Folch ratio 8:4:3 $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ and vigorously mixed. After centrifugation we applied the upper phase to a C_{18} clean-up cartridge, using 47:48:3 $\text{H}_2\text{O}/\text{MeOH}/\text{CHCl}_3$ for equilibration and washing. Glycolipids were eluted with 1 mL MeOH and dried under the N_2 stream.

To isolate the glycolipid fraction from *A. fumigatus* mycelium, we harvested it after growth for 2 days by filtration through a Miracloth filter (2.4.2). The cell wall and cell membrane were disrupted in a beads mill (BioSpec) with 0.5 mm glass beads in a volume of 15 mL of cold lysis buffer (200 mM Tris-HCl, 20 mM EDTA, pH 8, 1 mM PMSF). The lysate was centrifuged for 10 min at $10,000 \times g$ at 4°C to eliminate cell wall fragments. The supernatant was again centrifuged at $100,000 \times g$ (32,000 rpm) for 60 min. The pellet was resuspended in 20 mM Tris-HCl, 2 mM EDTA, pH 8 and kept at -80°C . The membrane pellet was resuspended in chloroform/methanol 1:1 to obtain a chloroform/methanol/ H_2O ratio of 10:10:3. For further homogenisation, we performed an ultrasonification in a water-ice-bath applying 3 x 15 pulses. Subsequently, the suspension was left for 1 hour in a sonication bath with intermittent mixing. After the following centrifugation at $10,000 \times g$ for 10 minutes and the re-extraction of the pellet, another centrifugation as described above, the supernatants were merged and dried under the N_2 stream.

For the saponification, we followed the same steps as described for the saponification in glycolipid extraction from spores.

The n-butanol partitioning was performed similar to the glycolipid extraction from spores. The glycolipids were resuspended in 1 mL butanol saturated with H_2O . Then, 1 mL of H_2O saturated with butanol was added and the suspension was vortexed, briefly centrifuged and the butanol phase was transferred to a clean falcon. The H_2O phase was re-extracted twice. We washed the collected supernatant with H_2O saturated with butanol and dried it under the N_2 stream. The glycolipid fractions were analysed by a mass spectrometry or HPTLC.

2.4.5 High-performance thin-layer chromatography (HPTLC)

HPTLC can separate different glycolipids depending on their solubility into the mobile phases.

We followed the method described by Toledo et al. (37). A glass chromatography tank was filled with solvent as mobile phase (chloroform/methanol/ H_2O [50:47:14 (v/v/v)] with 0.038% (w/v) CaCl_2), and the system was equilibrated for 2 hours. Glycolipid fractions were resuspended in 150 μL of solvent consisting of chloroform/MeOH/ H_2O 50:47:14 and 15 μL of each sample were applied to a 0.5 cm line drawn 1.5 cm parallel to the bottom of a lipid adsorbing silica gel HPTLC-plate using a microsyringe. The HPTLC plate was placed in the tank and left until the mobile phase front almost reached the top of the plate (approx. 45 min). Afterwards, the front line was marked and the silica membrane was dried under the fume hood. To visualise the glycolipids bands, we sprayed the silica plate with an orcinol/sulfuric acid reagent (0.5% orcinol (w/v), dissolved in 5.5% H_2SO_4 (v/v) in ethanol/water [8:3]) and developed it by heating the plate at 150°C until bands appeared.

The reaction of reducing sugars in glycolipids and glycosides with orcinol can be observed by a change of colour towards green-violet in case of pentoses, towards green-brownish in case of hexoses.

2.4.6 MALDI-TOF mass spectrometry

The glycolipid fractions were analysed by Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). For this purpose, each dried sample was re-suspended in ~200 µl of CHCl₃/MeOH/H₂O (30:60:8). 1 µL of 6-aza-2-thiothymine matrix solution (5 µg/mL in water) together with 2 µL of sample were spotted on a metal plate with the objective to obtain a homogenous analyte/matrix crystal. The matrix was evaporated by laser in a hot air stream, the molecules were released from the crystal and ionised. After acceleration of the ionised molecules in an electric field, the measurements were realised with a Voyager DE Pro work station (Applied Biosystems). The time-of-flight analysis is based on mass and valency and allows the determination of the mass-to-charge-ratio m/z [Da]. An ion detector transformed the incoming ions into electric signals which could be visualised as intensity peaks in a spectrum in dependence on the time-of-flight. This way, in the MALDI-TOF weight-related molecule species could be determined for each sample. The distinctive peak pattern for each molecule contains several peaks at intervals of 1 Da for the monocharged ion due to natural C¹³ distribution.

2.4.7 Infection of A549 carcinoma cells with *A. fumigatus* spores

A549 cell line is used as a simple pulmonary epithelial model of alveolar type II cells that grow as a confluent monolayer of adherent cells. Growth is possible by addition of DMEM and serum supplement like FCS. In order to analyse the impact of Af-3c on viability of cells, we infected A549 carcinoma cells with *A. fumigatus* spores of the fungal strains AfS35, derivate of D141, and ΔgntA. The latter is a knockout that is disabled in the zwitterionic GIPC pathway and does not express Af-3c. AfS35 was expected to produce Af-3c after germination as the glycolipid of interest is not present in spores (see 3.3.2). Direct mycel application would have been difficult to realise in terms of exact pathogen quantification in addition, the distinct mycel pathogenicity turns out to produce quickly vast cell damage which would put difficulties in the evaluation of the results.

2.4.7.1 Cell splitting and A549 cell growth

A549 carcinoma cells were grown in submersion in DMEM F12, 10% FCS, 37°C, 5% CO₂, for 2.5 days. For maintenance, cells were cultivated in small cell culture flask (690175, 50 mL, 25 cm²). When cells were confluent, they were washed with 3 mL PBS and after addition of 1 – 2 mL of trypsin/EDTA cell detachment was controlled under the microscope. The trypsin was diluted with 10 mL of DMEM F12, 10% FCS and cells were counted. For the different experiment set-ups, we seeded either 10⁵ cells per well in 1 mL of DMEM + 10% FCS in a 12-well-plate, or we seeded 2 x 10⁶ cells in 3 mL of medium in a small cell culture flask (690175, 50 mL, 25 cm²). Cells were grown for 2.5 days to >90% confluence monolayers. Medium was changed every 2 days.

2.4.7.2 Infection of A549 cells

The spore concentration was determined (see chapter 2.4.1) and the suspension diluted to different concentrations (around 10^5 spores/mL) in medium. This spore suspension was added to each well of a 12-well plate or to a 75 cm² culture flask as indicated in the result part. As reference served a negative control of A549 cells that were not infected but treated in the same way. The cells were incubated for 16 hours at 37°C, 5% CO₂. A death control was prepared by adding 1 mL ethanol per well or 1.5 mL to each small culture flask of A549 cells. After incubation, the cells were washed with PBS and survival evaluated by different methods.

2.4.7.3 Survival evaluation: cell counting

Viable cells that were still attached to the culture plate or flask were detached with trypsin/EDTA (500 µL per well or 1 mL per small culture flask) for up to 10 min under microscopical detachment control. 4.5 mL of DMEM + 10% FCS were then added to the cell suspension to stop the enzymatic activity. Cells were centrifuged 300 x g, 10 min, 4°C, and resuspended in 500 µL of PBS per well or flask. In a second experimental set-up, in order to avoid cell loss, dilution in stopping medium, centrifugation and concentration was avoided by direct counting of the cells in trypsin/EDTA after detachment. In both cases, the cells were combined in a 1:1 suspension with trypan blue and counted under the microscope in a Neubauer counting chamber as described in 2.4.1.

Our tests indicated that Calcein staining, an alternative method that is frequently used to measure cell viability or cytotoxicity in analogous bacterial infection protocols by hydrolysis of an acetoxymethyl ester in live cells and production of fluorescing Calcein, is not applicable for *A. fumigatus* pathogenicity as our pre-tests pointed to an unfavourable ester hydrolysis for the eukaryotic metabolism of the fungus.

2.5 Bioinformatic tools

The following programs were applied during this work:

SeqMan, Seqbuilder and MegAlign by DNASTAR for sequence analysis and primer/plasmid design.

BLAST® Basic Local Alignment Search Tool and NCBI gene bank prediction for sequence prediction and homology analysis.

TMHMM server v. 2.0 for transmembrane protein prediction by discrimination between soluble and membrane standing proteins using charge bias, hydrophobicity and helix length analysis.

Sisweb online for isotope distribution calculation.

3. RESULTS

3.1 Identification and characterisation of a UDP-GlcNAc transporter called UGT

3.1.1 The nucleotide sugar transporter UGT

Usually, nucleotide sugar (NS) synthesis takes place in the endoplasmic reticulum (ER), the cytosol or cell nucleus. Glycosylation processes instead are located in the ER and Golgi apparatus. Nucleotide sugar transporters (NST) are a group of highly conserved type III transmembrane proteins that provide the NS for glycosylation processes in the ER and Golgi apparatus. They work as antiporters that exchange a nucleotide sugar from the cytosol for the corresponding nucleotide monophosphate. Their highly hydrophobic structure with predominantly ten lipid bilayer crossing domains locates them in the ER or Golgi membrane. Topology analysis predicts a number of ten transmembrane domains though, also six to eleven transmembrane helices are known for some transporters.

However an even number is most common among these. *In vitro*, they are mostly monospecific but some show multiple substrate specificity (38). We could base our NST research on these available facts that are known characteristics for eukaryotic NST (figure 7).

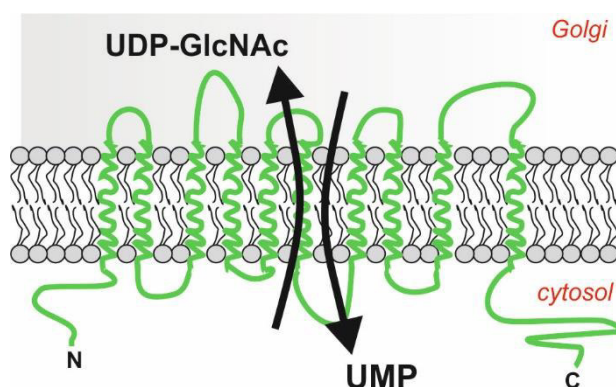


Figure 7: The putative UDP-GlcNAc transporter UGT has ten transmembrane helices and is assumed to transport UDP-GlcNAc into the Golgi apparatus.

3.1.1.1 Candidate protein

To begin, we needed an UDP-GlcNAc candidate protein. As Engel and colleagues characterised GntA, an enzyme essential for Af-3c production (see 1.5.3), as a N-acetylglucosaminyltransferase, the substrate for the Af-3c synthesis was known to be UDP-GlcNAc (29). A directed BLAST search for a UDP-GlcNAc transporter that would provide the NS for synthesis in the Golgi apparatus was performed. Interestingly, the gene AFUA_8G02090 encodes a putative NST, called here UGT, and is located adjacent to the *A. fumigatus* gene cluster AFUA_8G02040 to AFUA_8G02070 involved in the biosynthesis of Af-3c (see figure 8).

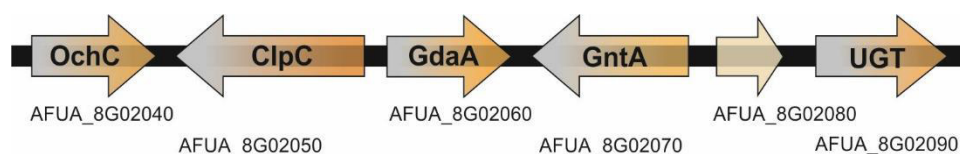


Figure 8: Gene cluster AFUA_8G02040 to AFUA_8G02090 on chromosome 8.

The gene AFUA_8G02090 has a length of 1077 bp, encoding for a protein of 359 amino acids, and a weight of 39 to 40 kDa similar to most NSTs. Moreover it lies directly downstream of the GlcNAc transferase GntA suggesting that it plays a role in Af-3c metabolism. The resemblance to SLC35A3, a member of the NST family responsible for UDP-GlcNAc transport (39), supported this hypothesis (see figure 9). In a distance tree, AFUA_8G02090 showed similarities to the encoding sequence of the SLC35 transporters' family member SLC35A. This subfamily contains highly conserved NSTs with specificity for CMP-sialic acid, UDP-galactose, and UDP-GlcNAc (39).

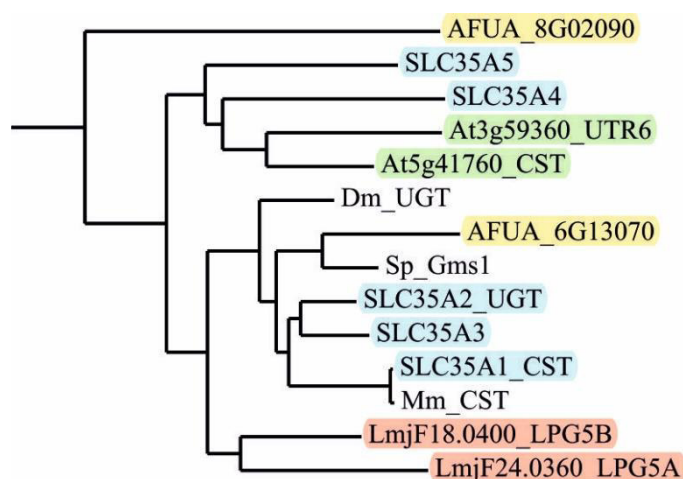


Figure 9: The phylogenetic tree of nucleotide sugar transporters shows phylogenetically close relationship between the members UGT and SLC35A.

3.1.1.2 cDNA synthesis of the AFUA_8G02090

cDNA synthesis allowed the analysis of a gene called AFUA_8G02090. cDNA was synthesised from mRNA that was obtained from *A. fumigatus* mycelium of the D141 strain (see 2.2.1). The nucleotide sequence was amplified via PCR using the primer pair JE83 // JE84 that matches with the restriction sites of BamHI and XbaI. They were designed based on the mRNA sequence of UGT, called XM_741904.1, predicted for the known *A. fumigatus* strain Af293 (https://www.ncbi.nlm.nih.gov/nuccore/XM_741904.1). After cloning in a plasmid, the amplified cDNA was sequenced which allowed its comparison to the predicted mRNA sequence as well as the transmembrane domain predictions for the encoded protein.

3.1.1.3 NCBI gene bank prediction for AFUA_8G02090

Sequencing of AFUA_8G02090 cDNA sequence displayed the *de facto* sequence as it revealed three important differences respective the predicted DNA sequence from mRNA.

In contrast to the predicted mRNA for XM_741904.1 (Af293), the sequence **ctggtc ctgcaaaatg cagcctcgat tatt**, highlighted in blue in figure 10 B. was not spliced although it was predicted as the first intron. Since this sequence contains 30 bp, the absence of splicing did not change the reading frame. In addition, the sequence **gtga gtgatttgct tcagcgcggg gttttcggag aatgcatcgc tgatgcggag gttggtag**, highlighted in red in figure 10 A., was predicted as an exon but actually corresponded to an intron. This splicing led to a shift in the reading frame so that 47 bp (underlined in figure 10 B.) were missing when using the primers JE83 // JE84. A new reverse primer JE102 containing the missing 47 nucleotides was designed to amplify the complete open reading frame displayed in figure 10 B.

Three independent PCR confirmed the UGT DNA sequence as presented in figure 10 B.

A.

```

1 atgcttcgag tgatgaacat atacaggttt agcttaacag ggcataccaa ggcccaagta
61 tcgacgcttg ccggtattct tctactacaa cataaaatcc agtcccagtc cacaaccgag
121 gacaagcact tcgatccact ggcaggcatc ctctctctcg aactcctcaa actgaccgtc
181 tcctcctctt gcatcgccca cacagcagaa gagccccaat ccctcctcgc aacctcctcaag
241 acaaaccacg aagaagccac cctcccagcc ctctcttaca ccgcccagtc cttcgcccaa
301 agcatcggtg cctccttctt cccctcctc caatacctca ccctctccca gaccaaatc
361 atcctcacc cactcctcgc aaccttctct ctcaaccaac gattcaccct ccagcactgg
421 acgtccaccc tgactatgac agcggggatc attctcgcgc agacaggcgc caacgccgca
481 gcagaaacgc agccccgcac cgcagccaac cccaacatcc acgcccagtc cctcctccca
541 ggcacccctg ccatgctgct ctctgggtcc tgcgtcgcac tgggaagcct ctccatcgaa
601 cgatcgctca agcgcgcgcg gaacaacaac accactacga ccgcgaaggg gagcgcgttc
661 ttcacccgca acgcacaact cgcgcgcgac agtctcctct ttgcaactct ctcgttcctc
721 tggaaggaaa agtgccgaat agacggatca cgcttcttgg aaggcttcaa tcgctgtgtc
781 tgggtgtttg tgggtgctca ggcgtccggt ggggttcctg tggcgtggtg tgtgagggcg
841 acgagttctg tgacgaagaa ctatgcgcag gggatggggg ttgcgattgc ggctgcgggg
901 ccgttggttg tcgagtcgaa ggatgttgat gggaaggtga gtgatttgct tcagcgcggg
961 gttttcggag aatgcatcgc tgatgcggag gttggttagc tcttgctggg gttgtgcttg
1021 tattgggggc ggtcgttggg tcggccttcg cagggcagag taTGA

```

1st variation:
intron that was
predicted as exon

B.

```

1 atgcttcgag tgatgaacat atacaggttt agcttaacag ggcataccaa ggcccaagta
61 tcgacgcttg ccggtattct tctactgggc ctgcaaaatg cagcctcgat tattctacaa
121 cataaaatcc agtcccagtc cacaaccgag gacaagcact tcgatccact ggcaggcatc
181 ctctctctcg aactcctcaa actgaccgtc tGcctcctct gcatcgccca cacagcagaa
241 gagccccaac cctcctcgcg aacctcctca acaaaccacg aagaagccac cctcccagcc
301 ctctcttaca ccgcccagtc cttcgcccaa agcatcggtg cctcctCctt cccctcctc
361 caatacctcG cctcctccca gacaaaactc atcctcacc cactcctcgc aaccttctc
421 ctcaaccaac gattcaccct ccagcactgg acgtccaccc tgGctatgac agcggggatc
481 attctcgcgc agacaggcgc caacgccgca gcagaaacgc agccccgcac cgcagccaac
541 cccaacatcc acgcccagtc cctcctccca ggcacccctg ccatgctgct ctctgggtcc
601 tgcgtcgcac tgggaagcct ctccatcgaa cgatcgctca agcgcgcgcg gaacaacaac
661 accactacga ccgcgaaggg gagcgcgttc ttcacccgca acgcacaact cgcgcgcgac
721 agtctcctct ttgcaactct ctcgttcctc tggaaggaaa agtgccgaat agacggatca
781 cgcttcttgg aaggcCtcaa tcgctgtgtg tgggtgtttg tgggtgctca ggctgcgggt
841 ggggttcttg tggcgtggtg tgtgagggcg acgagttctg tgacgaagaa ctatgcgcag
901 gggatggggg ttgcgattgc ggctgcgggg ccgttggttg tcgagtcgaa ggatgttgat
961 gggaaagctt ttgtcggggt tgtgcttgta ttggggggcg tcgttggtgc ggccttcgca
1021 gggcagagta tgacgggaac caatgtgacg gagaagaggg aagtc aaagg tgtggtgTAG

```

2nd variation: exon
that was predicted
as an intron

3rd variation:
ending sequence
that lay behind the
assumed stop
codon

Figure 10: Comparison of the predicted mRNA sequence (A.) and sequenced cDNA (B.). The actual UGT sequence lacks a NS subsequence that was predicted as an exon and shows an additional exon that was not predicted. The two mispredictions lead to wrong sequence and to a frame shift which provided an actual stop codon that lay behind the predicted one.

3.1.1.4 Protein transmembrane domain prediction

For a better understanding of the discovered transporter UGT, transmembrane domains were examined. The TMHMM 2.0 probability prediction of transmembrane helices for the cloned D141 cDNA sequence for AFUA_8G02090 showed 10 hydrophobic domains of which some show low hydrophobicity in a typical pattern as seen in many NSTs (see figure 11). NSTs are highly conserved type III transmembrane proteins and, as known to date, they usually present an even number of transmembrane domains (TMD).

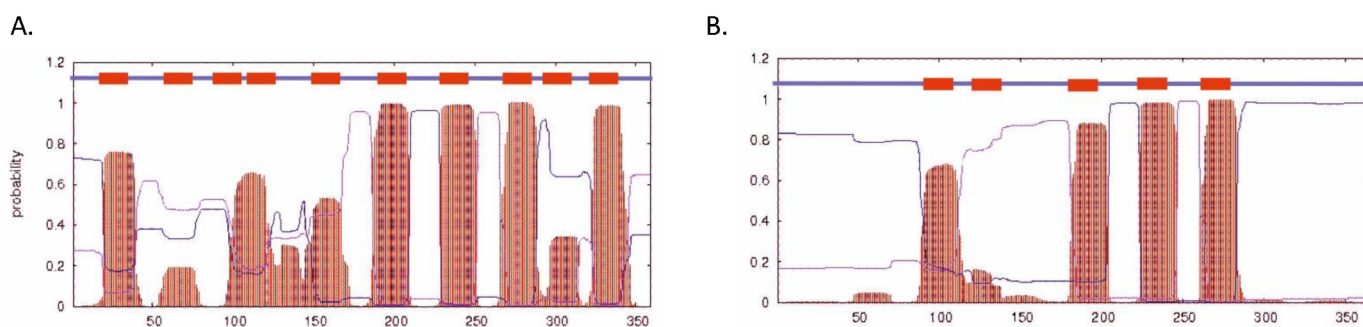


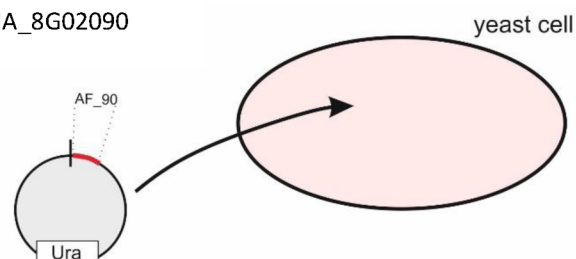
Figure 11: Differences in the TMD probability prediction based on sequenced cDNA (A.) and predicted mRNA annotated in the database (XM_741904.1) (B.). The predicted mRNA based amino acid sequence would encode around five highly hydrophobic domains whereas the cDNA deduced sequence encoded for up to ten transmembrane domains which is more likely for a transporter belonging to the NST family.

Figure based on original pattern TMHMM server v. 2.0.

3.2 UGT expression in yeast

To provide an *in vitro* model for UGT analysis, the transporter was expressed in yeast, an organism that lacks endogenous UDP-GlcNAc transport. This strain may also serve for further glycosyltransferases expression for Af-3c production and analysis.

pYESCup1_Ura with
AFUA_8G02090



3.2.1 First yeast construct: FLAG tag

3.2.1.1 Cloning of a FLAG tagged UGT encoding plasmid and a SLC35A3 encoding plasmid as positive control in the yeast expression vector pYES_URA

The primer pair JE83 // JE102 was used for amplification of the gene AFUA_8G02090. The fragment was inserted in the multiple cloning site (MCS) of the *S. cerevisiae* expression vector pYESCup1 using the BamHI and XbaI restriction sites. The pYESCup1 vector enables expression of N-terminally tagged proteins under the copper inducible promoter and contains the *URA3* gene, providing prototrophy for uracil for selection purpose (see 2.3.2).

JE83	gcaa <u>GGATCC</u> CTTCGAGTGATGAACATATACAGG (BamHI)
JE102	gcag <u>TCTAGAC</u> TACACCACACCTTTGACTTCCCTCTTCTCCGTCACATTGGTT CCCGTCATACTCTGCCCTGCGAAG (XbaI)

The characterised human UDP-GlcNAc transporter SLC35A3 protein served as our positive control. Analogously to the UGT cloning, the SLC35A3 sequence was inserted in the pYEScup1 vector via the BamHI and XbaI restriction sites to enable expression of the N-terminally FLAG tagged protein.

3.2.1.2 Recombinant protein expression of FLAG tagged UGT and FLAG tagged SLC35A3

UGT has a length of 359 amino acids and a calculated molecular mass of 39.7 kDa whereas SLC35A3 has a length of 339 amino acids and a weight of 37.5 kDa.

The plasmids encoding FLAG tagged UGT and SLC35A3 were transformed in *S. cerevisiae* K03 strain and single clones were selected on agar plates lacking uracil. Clones were grown in liquid cultures and protein expression was induced with CuSO₄ (0.5 mM, see 2.3.5) for variable duration (t(0) to t(2) as 0, 3 and 6 hours). To avoid problems due to overexpression of this extremely hydrophobic protein and preserve the organelles' structure, expression had to be controlled with restricted inductor concentration and short incubation times. The yeast cells were mechanically lysed, the total protein concentration was measured in a BCA assay and expression of the protein was analysed by Western blot. Several primary antibodies including the monoclonal mouse anti-FLAG M5 antibody (see figure 12) and anti-FLAG M2 antibody (data not shown) were tested in combination with fluorescent 680 CW antibodies and visualised with a LI-COR imager. Unfortunately, the high background did not allow observing expression of the proteins. Despite the different nature and size of the UGT and SLC35A3 transporters, the same pattern can be observed. Attempts to increase expression by extending the induction time up to 22 hours (t(3)) or increase the CuSO₄ concentration up to 1.5 mM did not intensify the FLAG-transporter signals above the background.

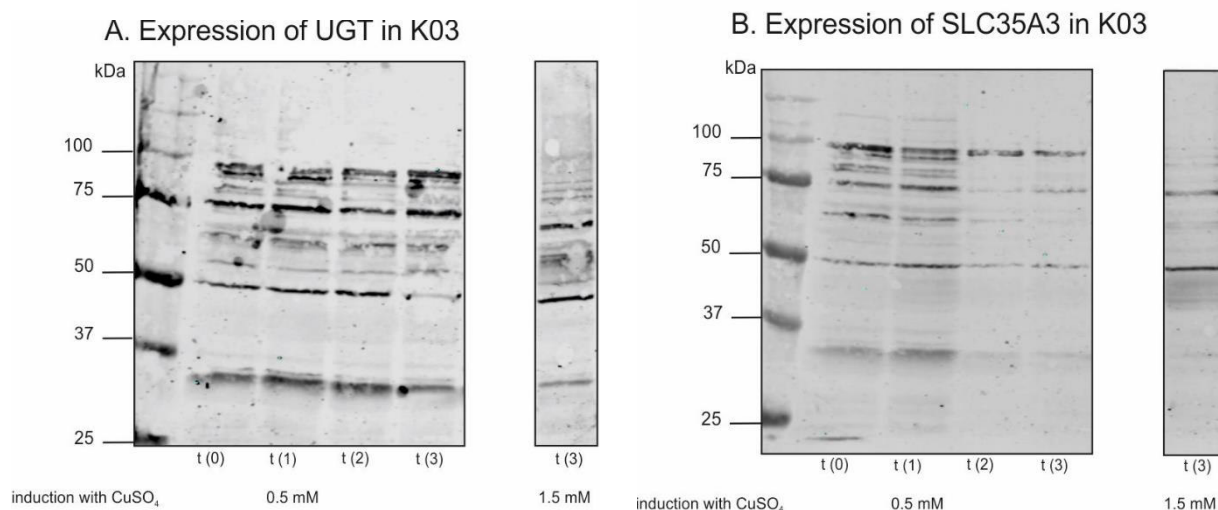


Figure 12: Expression of UGT (A.) and SLC35A3 (B.) in K03. The similar signal pattern for UGT and SLC35A3 despite different protein size suggests high background activity. Visualisation of UGT mono- and dimer form (around 37 and 87 kDa) is questionable for the strong signal overlap. Also, increasing expression over time cannot be observed. Higher inducer concentration did not produce higher expression yields.

In order to reduce background signal, we repeated the SDS-PAGE/Western blot with aliquots obtained in the same protein expression procedure by applying a monoclonal Anti-FLAG M2 antibody produced in rabbit, but it produced only poor signal with, still, high background activity.

3.2.1.3 Golgi-rich fraction of FLAG tagged UGT and SLC35A

In order to avoid the described high background signal, we prepared a Golgi-rich fraction of *S. cerevisiae* expressing FLAG-UGT. But in the SDS-PAGE/Western blot we saw low transporter expression. Unfortunately, we could not increase concentration of CuSO_4 since concentrations higher than the used one (0.5 mM) are toxic (see 3.2.1.2).

3.2.2 Expression of V5 tagged UGT and SLC35A3 in yeast

3.2.2.1 Cloning of a V5 tagged UGT encoding plasmid and a SLC35A encoding plasmid as positive control in the yeast expression vector pYES_URA

To improve detection of the transporter after expression, we decided to re-clone the proteins of interest with a V5 tag. The FLAG tag in the pYESCup1 vector was replaced by a V5 tag using the HindIII and KpnI restriction sites. The V5 tag adapter sequences were

5'-AGCTTcgaATGGGTAAGCCTATCCCTAACCCCTCTCCTCGGTCTCGATTCTACGggtacAGCTT
cgaATGGGTAAGCCTATCCCTAACCCCTCTCCTCGGTCTCGATTCTACGggtac-3'
(MH178 linker)

and

5'-cCGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCCATtcgA-3'
(MH179 linker).

For the ligation, we used the KpnI and XbaI restriction sites for UGT sequence and KpnI and XhoI for the SLC35A3 sequence to place the sequence of interest C-terminally to the tag. The UGT encoding sequence (AFUA_8G02090 gene) was amplified with the primer pair FR208 // FR209 and, the SLC35A3 encoding sequence with FR210 // FR211.

FR208	ctga <u>GGTACC</u> CTTCGAGTGATGAACATA (KpnI)
FR209	catg <u>TCTAGA</u> CTACACCACACCTTTGAC (XbaI)
FR210	ctga <u>GGTACC</u> TTGCGCAACCTAAAATACG (KpnI)
FR211	catgTCTAGA <u>CTCGAG</u> aCTATGCT (XhoI)

3.2.2.2 Recombinant protein expression of V5 tagged UGT and V5 tagged SLC35A3 in yeast

As was described previously, we transformed the plasmids in *S. cerevisiae* K03 strain (BY4741 with YEL004W::KanMX4) as well as in the wild type reference strain BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0).

The protein expression protocol was followed as described for the FLAG tagged proteins (see 2.3.5). After induction with CuSO₄, an aliquot was harvested after 0, 3 and 6 hours for each plasmid and each strain. Cells were lysed and the protein of interest could be detected performing an SDS-PAGE/Western blot. We applied a monoclonal anti-V5 antibody produced in mouse as 1st antibody and an anti-mouse IR Dye 800 CW as 2nd antibody.

In this test expression, the background signal could be reduced to a low level. At time point (3), both in the K03 as well as in the reference strain, a band of a molecule around 70 to 80 kDa could be detected (see figure 13). This likely represents the dimer form of UGT which has a monomer molecular mass of 39.7 kDa. Unexpectedly, the control transporter SLC35A3 could not be detected in the LI-COR fluorescence spectrometry.

A. Reference strain BY4741

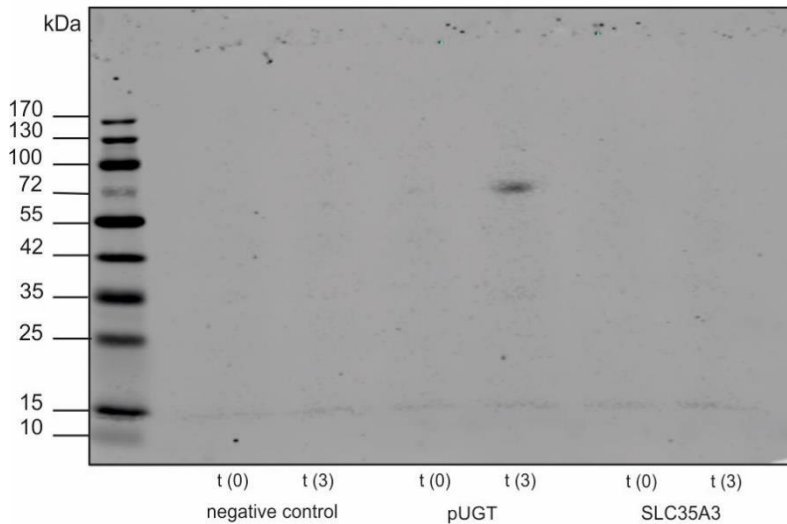
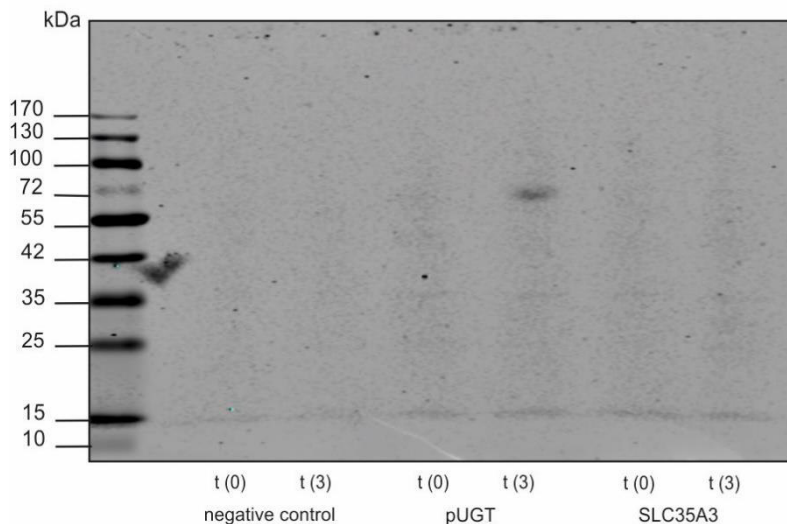


Figure 13: NST expression of a negative control, UGT and SLC35A3 in two different strains. Both show moderate UGT expression after t (3) = 6 hours of induction time. A. BY4741 strain; B. K03 strain.

B. K03



3.2.3 *In vitro* transport assay with UGT containing Golgi-rich fractions

In *Aspergillus fumigatus*, synthesis of the cell membrane glycolipid Af-3c requires the metabolite UDP-GlcNAc and takes place in the lumen of the Golgi apparatus (29) (see 1.5.3). Thus we assumed that UGT must be present in the Golgi apparatus of transformed yeast as well and so we used Golgi-rich fractions isolated from the yeast cells to measure transport activity to show that NS transport is only given in UGT transformed yeast. Therefore, the plasmid encoding UGT described in 2.2 and empty vector (as negative control) were transformed into *S. cerevisiae* strain K03 and single clones were isolated. After growth and induction of protein expression for 3 hours, the yeast cell wall was enzymatically removed and the plasma membrane of the resulting spheroplasts was gently lysed by dounce homogenisation. The Golgi-rich fraction was then obtained by differential centrifugation (see 2.3.3). SDS-PAGE and Western blot confirmed presence of the transporter in the Golgi-rich fraction.

The technical difficulty of this assay lies in the obtaining of sealed and thus transport competent microsomes, which requires a very mild disruption of spheroplasts. To test the integrity and transport competence of the Golgi vesicles prepared, we measured UDP-Glc transport in Golgi fraction isolated from yeast expressing UGT and yeast transformed with the empty vector. Indeed yeast Golgi vesicles show a clear endogenous transport of UDP-Glc and GDP-mannose whereas transport of other nucleotide sugars such as UDP-GlcNAc is low or absent.

For the transport assay, Golgi-rich fractions containing or not UGT, were incubated for 30 s with radiolabeled UDP-[¹⁴C]-GlcNAc or UDP-[6-³H]-Glc. The incubation allows the uptake of the nucleotide sugar against the corresponding nucleotide monophosphate naturally present in the vesicles. Then, vesicles were filtered on a nitrocellulose membrane and the excess of radioactive nucleotide sugar was washed before the radioactivity associated with the vesicles is measured in a scintillation counter.

The specific activity [dpm/pmol] of the used radioactive nucleotide sugar solution was defined by measuring the radioactivity in 2 µL solution. To calculate the specific activity, the measured decay per minutes (dpm) was multiplied by 25 (since 50 µL of this solution were used per assay) and divided by 100 (since each assay contained 100 pmol nucleotide sugar). The assay was performed with 100 µg protein for 30 s so that the transport activity could be calculated per pmol of NS, per min, for 1 mg of protein with the following formula:

$$(\text{activity [dpm]}_{\text{sample}} / \text{specific activity [dpm/pmol]}) \times 20 \\ = \text{transport in [pmol/mg/min]}$$

A.

³ H	cpm (2μL, 30 sec)	dpm 60%	specific activity (dpm/pmol)	mean value reference of specific activity
2μL UDP-Glc	21174	35290	8822.5	8822.09
	21172	35286.7	8821.68	
			pmol/mg/min protein	
UGT	834	1390	3.15	
	852	1420	3.22	

B.

¹⁴ C	cpm (2μL, 30 sec)	dpm 95.6%	specific activity (dpm/pmol)	mean value reference of specific activity
2μL UDP-GlcNAc	6047	6325.31	1581.25	1591.23
	6123	6404.81	1601.2	
			pmol/mg/min protein	
UGT	576	602.51	7.57	
	551	576.36	7.24	
Negative control	63	65.9	0.83	
	61	63.81	0.8	

Table 5: Transport assays. Calculation of transport activity. A. In UGT containing vesicles, transport of UDP-Glc at low levels (3.19 pmol/mg/min protein) could be detected which suggested endogenous NS transport in the yeast strain because UGT does not transport XDP-Glc. B. UGT containing vesicles show UDP-GlcNAc transport at intermediate levels (7.45 pmol/mg/min protein) whereas the mock transfected yeast Golgis show insignificant levels of UDP-GlcNAc transport activity (< 6 pmol/mg/min protein).

We performed the assay with sample duplicates. In UDP-Glc transport assay, we detected a transport activity both in the UGT containing as well as the negative control sample, which indicated transport competent Golgi membranes and sealed vesicles.

Transport of UDP-GlcNAc, the specific target of UGT, was present in UGT containing Golgi vesicles compared to the negative control, as describes in table 5 B.

UGT containing microsomes transported **7.45 pmol of UDP-GlcNAc/mg/min**, whereas in the negative control, a transport of only **0.825 pmol of protein/mg/min** was measured (see figure 14).

We repeated the vesicle preparation and Golgi transport assay but could not replicate the result. We suspected a general methodological problem, as the BCA of the same yeast construct showed appropriate protein concentration.

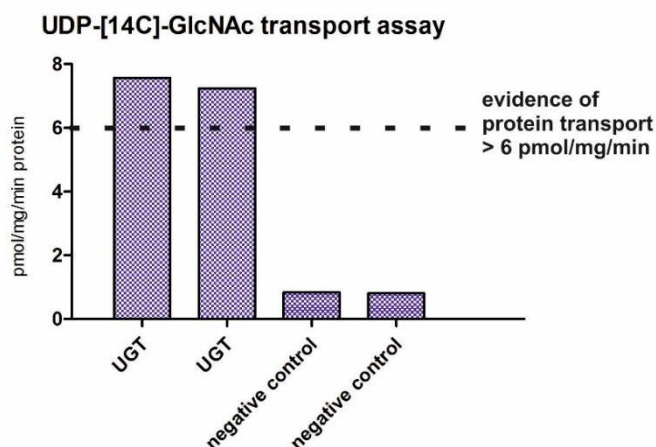


Figure 14: UDP-GlcNAc transport activity of UGT and mock transfected yeast Golgi preparations. Radiolabeled substrate GlcNAc-UDP served as marker (n = 1).

3.3 Characterisation of Af-3c glycolipid in *A. fumigatus*

To analyse the function of Af-3c, several *A. fumigatus* strains are available. The *A. fumigatus* AfS35 strain is a derivative of the clinical isolate D141 that lacks the *akuA* gene (involved in the non-homologous end joining pathway) and is here designated as wild type strain. This strain expresses both acidic glycoinositolphospholipids (GIPCs) and the zwitterionic GIPC Af-3c (37,26). The Δ gntA strain is deficient in the N-acetylglucosaminyltransferase GntA that initiates Af-3c biosynthesis and thus expresses only acidic GIPCs (29). This strain was complemented by reintroducing *gntA* at its original locus to generate the Δ gntA-reintro strain (29). In contrast, the Δ MitA strain lacks the α -(1,2)-mannosyltransferase initiating the synthesis of all acidic GIPCs, and hence contains Af-3c as sole GIPC (30).

Since *Aspergillus* is a ubiquitous fungus spread by airborne spores, contamination of cultures and even stocks is possible. Thus before analysing the effects of Af-3c presence or absence in different assays, we screened the used clones by 1st) verifying sensitivity and resistance to antibiotics and 2nd) analysing the GIPC profile of the different strains (see 2.4.3).

Growth on agar plates containing phleomycin or hygromycin confirmed resistance of the Δ gntA strain to phleomycin and sensitivity to hygromycin, whereas Δ MitA was resistant to hygromycin and sensitive to phleomycin as expected due to genetic resistance. In contrast, several clones of the Δ gntA-reintro had to be eliminated since they displayed resistance to both hygromycin and phleomycin although this strain should be only hygromycin resistant.

As described in 2.4.4, we extracted the glycolipid fraction from spores of Δ MitA and mycelia of the different strains and analysed the glycolipids by HPTLC and mass spectrometry.

3.3.1 High Performance Thin Layer Chromatography (HPTLC)

We started glycolipid analysis by HPTLC. In a previous study, Toledo and collaborators described a method for the separation of *A. fumigatus* GIPCs by HPTLC. The authors obtained a separation of GIPCs containing two, three or four hexoses and of Af-3c using a mobile phase consisting of chloroform/methanol/water in a 50/47/14 ratio. Carbohydrates were then stained by orcinol (37). This method was applied to the glycolipid fractions that we extracted from *A. fumigatus* S35, Δ MitA and Δ gntA strains (figure 15). In contrast to Toledo et al., and despite repeated attempts, the glycolipids were only separated into two broad bands. In absence of GIPC standards, we tentatively assigned them to a mixture of Hex₂-IPC and Hex₃-IPC on one hand (band A) and Hex₄-IPC and Af-3c (Hex₂GlcN-IPC) on the other hand (band B). Also, a low-mobility band can be observed in Δ gntA which might correspond to a Hex₃-IPC substituted by a phosphocholine group as described by Simenel and colleagues (26).

HPTLC using an alternative solvent system – CHCl₃:MeOH [1:4] (solvent A) followed by CHCl₃:MeOH:H₂O(0.02%CaCl₂) [30:18:4] (solvent B) – did not allow a clear separation of Af-3c either. HPTLC was thus not suitable to assess the GIPCs profile of the strain.

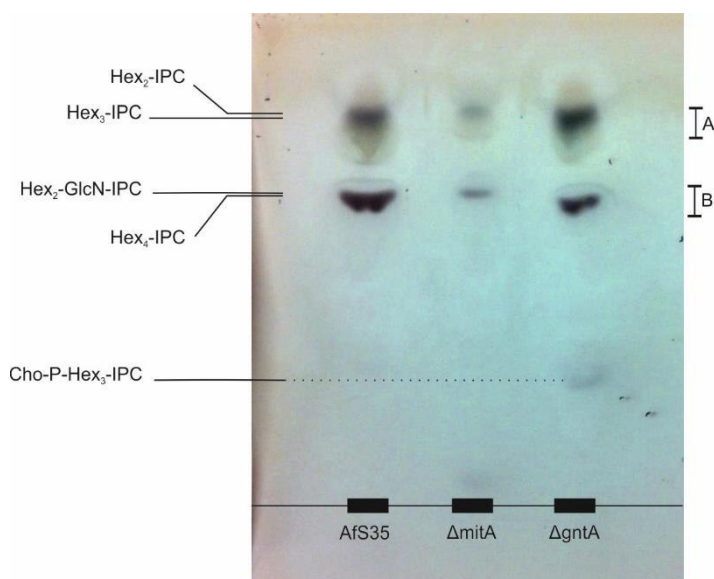


Figure 15: HPTLC development that shows different GIPC profiles for wildtype, Δ mitA and Δ gntA strain. Af-3c containing band (Hex₂GlcN-IPC) is overlapping with Hex₄-IPC.

3.3.2 MALDI-TOF mass spectrometry

For a differentiated glycolipid profile analysis we applied mass spectrometry. GIPCs isolated from AfS35, Δ gntA, Δ MitA and Δ gntA-reintro mutant were analysed in negative ion mode in Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Due to variations in the phytosphingosine base and the fatty acid chain constituting the ceramide, four peaks were observed for IPC. Phytosphingosine base with 18 or 20 carbons (t18:0 and t20:0) were bound to monohydroxylated saturated (h24:0) or to dehydroxylated saturated (d24:0) lignoceric acid (C₂₄H₄₈O₂). h24:0 molecules represented the main fatty acid group whereas d24:0 compounds represented the minor GIPC fraction for each species. Each of these four IPC species can be substituted by a mannose residue to give rise to MIPC. Further extension by mannose or galactofuranose residues yield Hex₂-IPC and Hex₃-IPC. In addition to these acidic glycolipids, IPC can be extended by a glucosamine and two mannose residues to generate Af-3c (Hex₂-GlcN-IPC).

The mass peaks of these different glycolipids are indicated in the figure below (figure 16) and can be observed in the glycolipids fraction obtained from wild type *Aspergillus* AfS35.

Unluckily, the masses of Hex₂-GlcN-IPC and Hex₃-IPC only differ by 1 Da, also each compound gives rise to a series of peaks differing by the same mass (1 Da) due to the natural occurrence of carbon 13 (¹³C). A characteristic pattern can be seen in the insert showing the MS peaks between 1400 to 1500 in figure 17, A. and C. This complicated the interpretation of the data. Nevertheless, comparison of the spectra from AfS35, ΔgntA and ΔgntA-reintro showed absence of Af-3c in the ΔgntA strain as seen by absence of the peak at 1409 and re-expression in the ΔgntA-reintro strain (compare right-sided spectrum details of figure 17, A. to C.).

In the ΔgntA-reintro strain Hex₂-GlcN-IPC synthesis was lower as in wild type as shown by the ratio between the peaks at 1409 and 1410 as previously reported (29).

We also extracted the glycolipid fraction from resting spores of ΔMitA knockout strain but we could not detect the presence of zwitterionic GIPC.

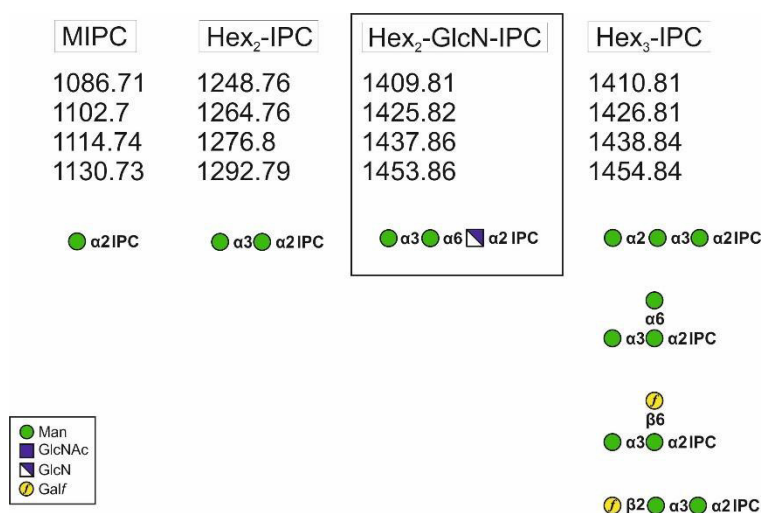
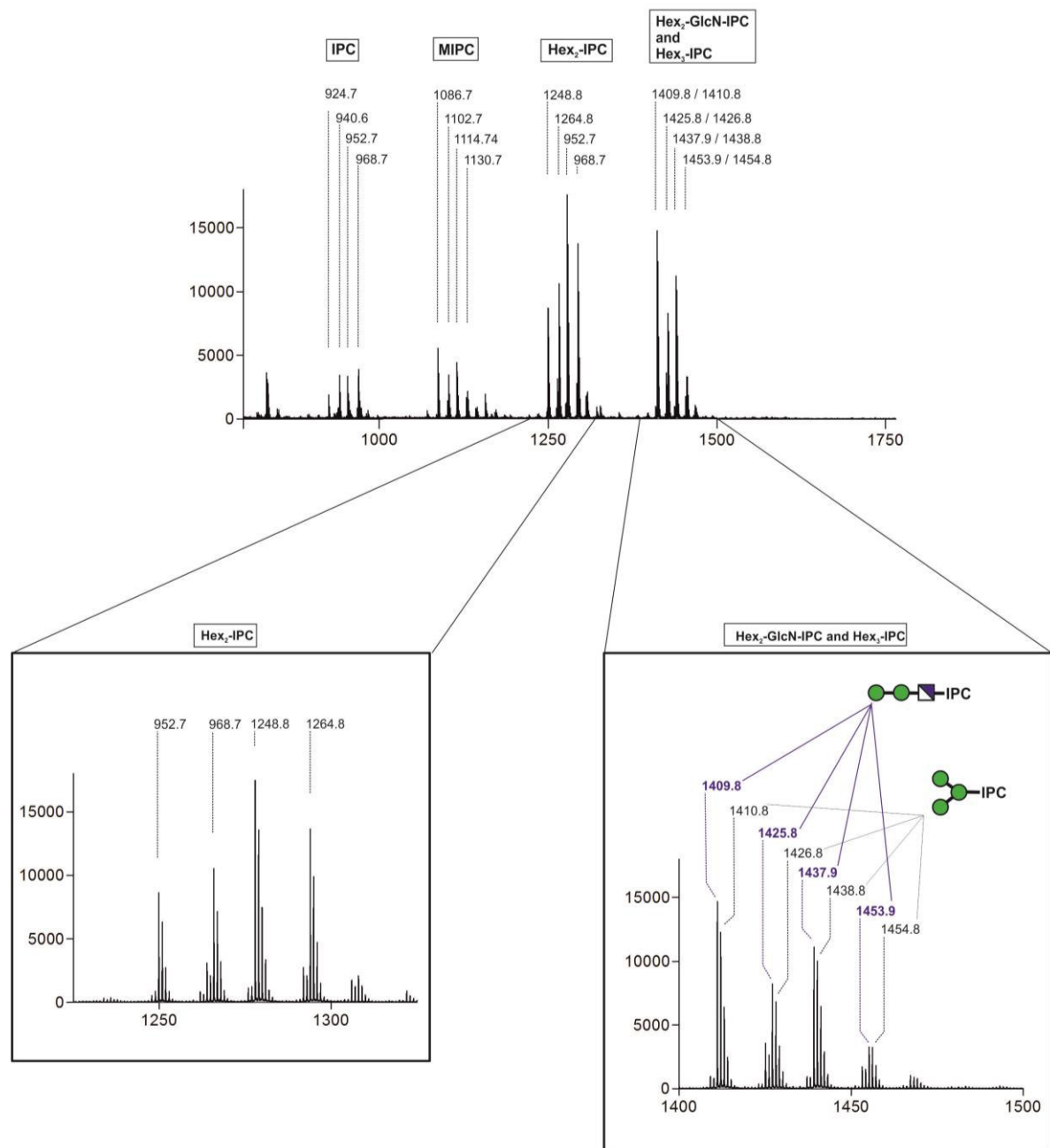


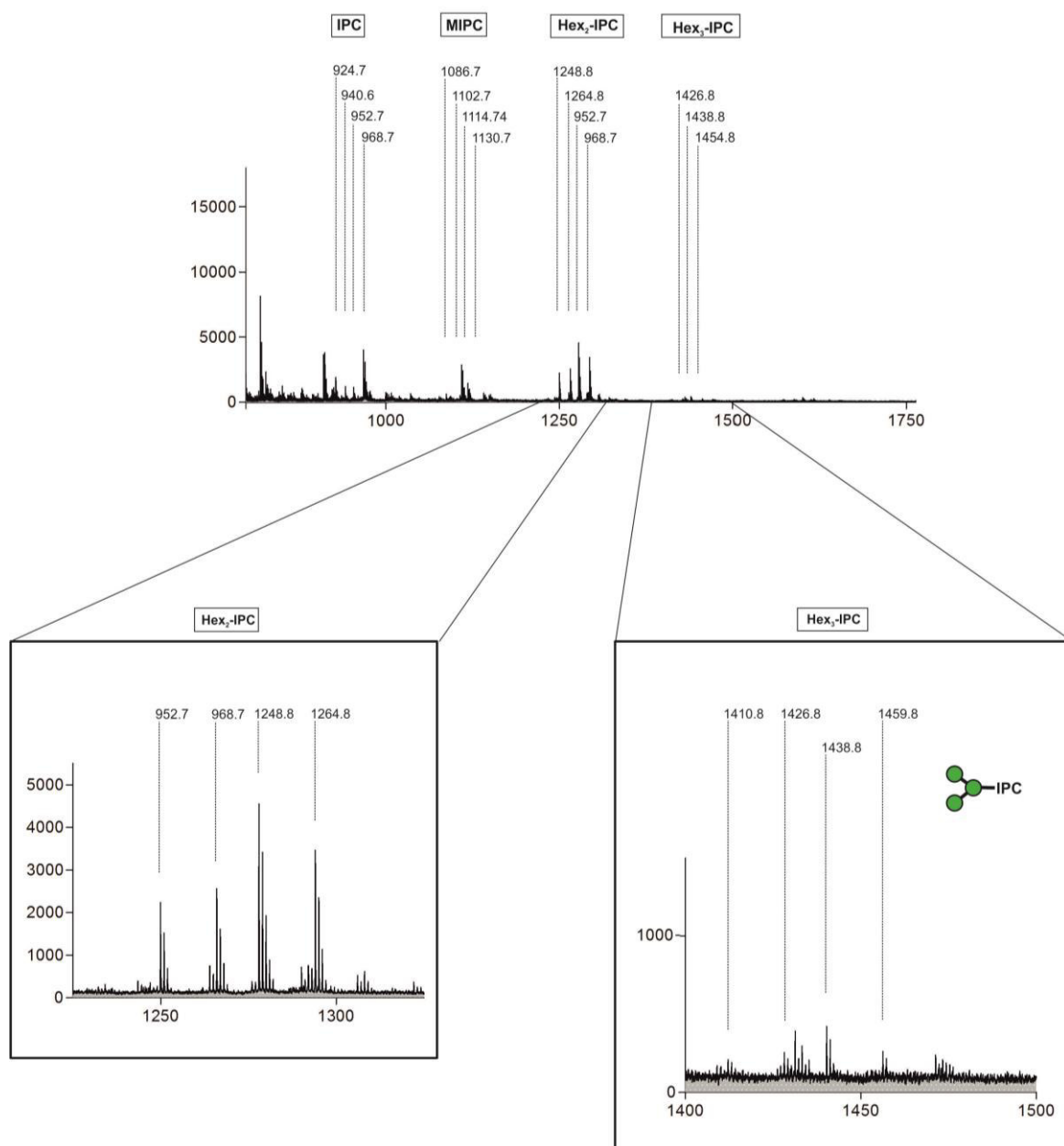
Figure 16: The figure shows the mass main peaks as known for the different GIPCs. Mass peaks of Hex₂-GlcN-IPC and Hex₃-IPC differ by only 1 Da.

A. MALDI-TOF glycolipid profile of AfS35 extracted from mycel



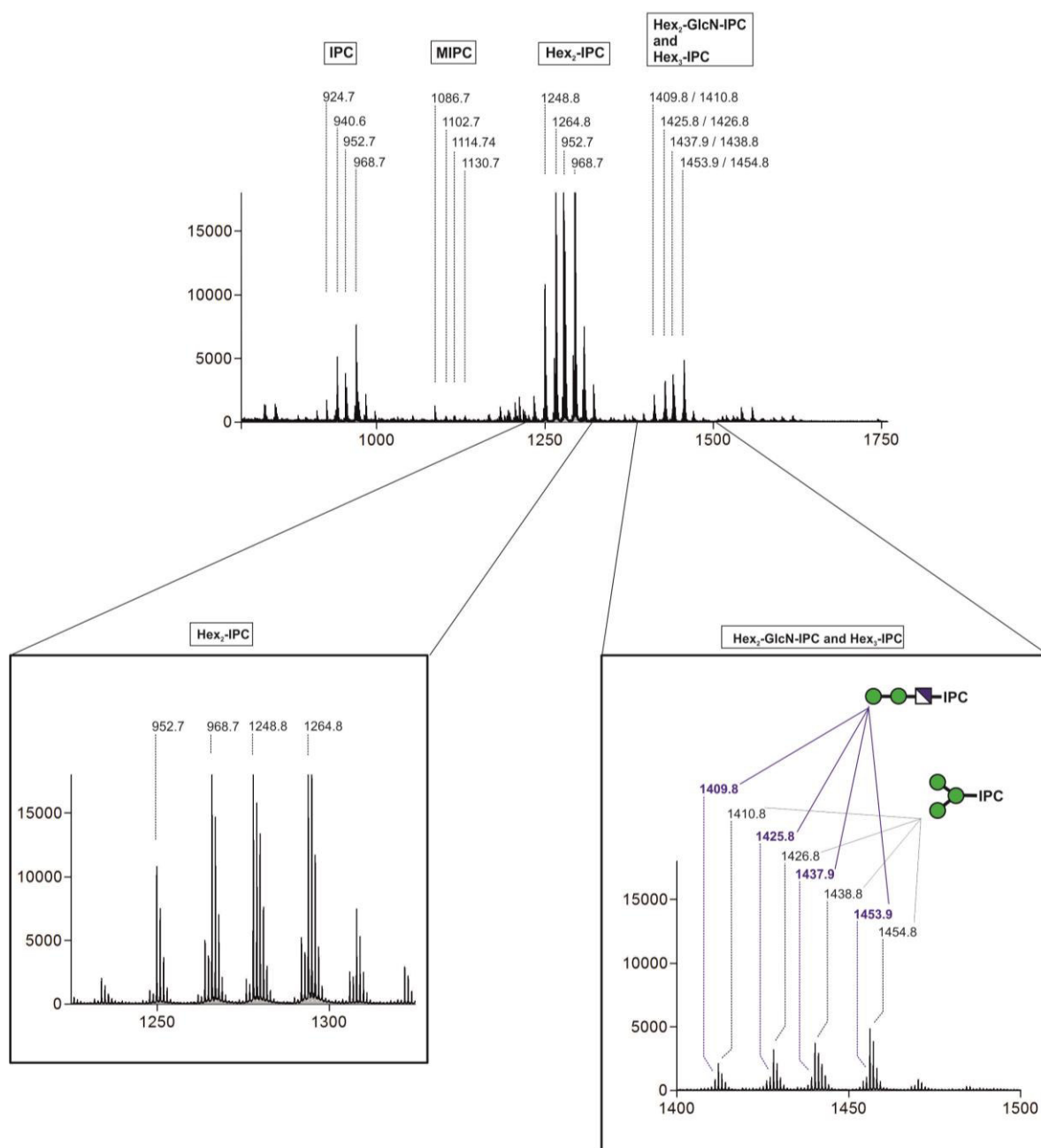
A. MALDI-TOF MS spectrum showing the glycolipid profile of wildtype AfS35 extracted from mycelium by homogenisation, centrifugation, saponification, BuOH extraction, C₁₈ clean-up and drying under N₂ stream: MIPC precursor Hex₂-IPC production at high levels, overlapping mass peak patterns of Hex₂-GlcN-IPC (Af-3c) and Hex₃-IPC at high levels.

B. MALDI-TOF glycolipid profile of Δ gntA extracted from mycel



B. MALDI-TOF MS spectrum showing the glycolipid profile of mutant Δ gntA extracted from mycelium by homogenisation, centrifugation, saponification, BuOH extraction, C₁₈ clean-up and drying under N₂ stream: MIPC precursor Hex₂-IPC production at normal levels, mass peak patterns of Hex₂-IPC and Hex₃-IPC production at normal levels, Hex₂-GlcN-IPC (Af-3c) production not detectable.

C. MALDI-TOF glycolipid profile of $\Delta gntA$ -reintro extracted from mycel



C. MALDI-TOF MS spectrum showing the glycolipid profile of mutant $\Delta gntA$ -reintro extracted from mycelium by homogenisation, centrifugation, saponification, BuOH extraction, C₁₈ clean-up and drying under N₂ stream: MIPC precursor Hex₂-IPC production at high levels, mass peak patterns of complex glycolipids overlap: Hex₂-GlcN-IPC (Af-3c) production at low levels and Hex₃-IPC production at intermediate levels.

Figure 17, A - C: MALDI-TOF analysis of GIPC fractions isolated from mycelium of AfS35, Δ gntA, and Δ gntA-reintro. Each GIPC species was represented by four different compounds due to variations in the ceramide moiety and in the fatty acid moiety (minor compounds: t18:0/d24:0 and t20:0/d24:0; main compounds: t18:0/h24:0 and t20:0/h24:0). Each compound, respectively, showed a multi-peak pattern due to steady C^{13} distribution where each C^{13} increased the mass of 1 Da. Different intensities resulted from variations in the accumulated quantity of each compound. The characteristic peak pattern in the range of 1400 to 1460 suggests overlapping profiles of the mass-similar proteins Hex₂-GlcN-IPC and Hex₃-IPC in wildtype and complemented strain. Af-3c production was lower in reconstituted mutant in comparison to the wildtype strain.

3.4 Determination of the pathogenicity of Af-3c using AfS35 and Δ gntA

In humans, the lung epithelium is the first tissue to be exposed to *A. fumigatus*, so, conventional pathophysiological course of *A. fumigatus* infection takes place beginning from this point of entry. In the second part of the Af-3c study, our efforts aimed at establishing an *in vitro* model that imitates contact of the pathogen to lung epithelium after inhalation of spores and that reflects the fungal virulence. Such assay would allow selecting mutants to be tested in other virulence models (such as murine infection model) in the future and thereby contribute to limit animal studies.

Our aim was to establish an assay to evaluate cell damage due to *A. fumigatus* infection by quantification of cell survival comparing *A. fumigatus*-infected to non-infected cells as well as comparing cells that were incubated with Af-3c producing to Af-3c deficient *A. fumigatus* strains.

3.4.1 Infection of A549 carcinoma cells with AfS35 and Δ gntA spores

In our study, the A549 carcinoma cell line monolayers served as alveolar type II cell model and were infected with different concentrations of AfS35 and Δ gntA spores. Several experiments were performed in order to determine an appropriate multiplicity of infection (MOI), adequate incubation time and proper evaluation method. For example, Calcein staining like other methods that are commonly used in infection models could not be used due to the eukaryotic metabolism of fungi. For each experiment, a control with uninfected A549 cells was used as a measure of 100% survival (equalised with no cell damage).

Experiments were carried out with fresh spore suspensions of defined concentration. The concentration was determined by optical density measurement of various dilutions and by plating diluted spore suspensions and counting the colony forming units (CFUs). In initial experiments, we grew A549 cells in 6-wells or 12-wells culture plates. Unfortunately, the cells did not detach by simple trypsin/EDTA treatment and required cell scraping, which created significant cell damage. Therefore, we opted for T25 flasks in subsequent experiments. Since the flasks are closed, it was possible to ease detachment by tapping the flask after trypsin/EDTA treatment. Cells were grown in F12-DMEM medium with 10% FCS until ~90% confluency. They were washed with medium without FCS and infected with fungal spores suspended at the appropriate concentration in medium without FCS. The fungal spores require approximately 8 hours to form germ tubes that will express Af-3c. Overnight incubation was thus chosen.

After 16 or 24 h of co-culture, adherent cells were washed with medium to eliminate dead cells and fungal hyphae and the remaining cells detached by trypsin/EDTA were counted in a Neubauer chamber.

As describes in 2.4.7, technical duplicates, triplicates or quadruplicates were performed to scale down errors in measurement.

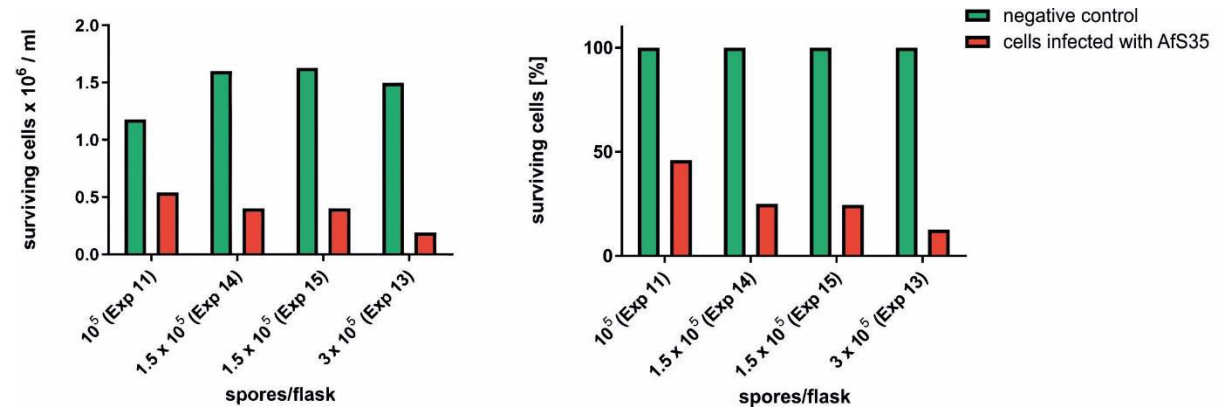
After evaluation of the experiments, in order to consolidate the data, another experiment with a setting identical to experiment 14 was carried out by members of Prof. Routier's lab team and the result kindly put at my disposition (experiment 15).

As shown in table 6, we could show that after infection with AfS35 spores the number of live A549 cells was reduced in comparison to the non-infected cells control. An infection dose of 10^5 spores/flask enabled to observe ~50% cell damage (experiments 11 and 12). Moreover, cell damage was proportional to the number of fungal spores used for infection (experiments 12, 13, 14 and 15). Higher cell damage (lower cell survival) was observed with 3×10^5 spores/flask in comparison to 1.5×10^5 spores or 10^5 spores.

Experiment number	Spore concentration per sample	Number of samples	Maximum incubation time	Counting method	Uninfected control [x 10^6 cells/mL]	Surviving cells after infection with AfS35 spores [x 10^6 cells/mL]	Surviving cells after infection with Δ gntA spores [x 10^6 cells/mL]
5	4×10^4 spores/mL	triplicate	16 h	Counting after centrifugation and resuspension	0.2	0.16 (79%)	0.19 (95.3%)
11	10^5 spores/flask	triplicate	24 h	Direct counting	1.18	0.54 (46%)	--
12	10^5 spores/flask	triplicate	16 h	Direct counting	2.64	1.11 (42%)	--
13	3×10^5 spores/flask	triplicate	16 h	Direct counting	1.5	0.19 (12.7%)	0.23 (15.7%)
14	1.5×10^5 spores/flask	triplicate	16 h	Direct counting	1.6	0.4 (25%)	0.48 (30%)
15	1.5×10^5 spores/flask	triplicate	16 h	Direct counting	1.63	0.4 (24.5%)	0.48 (29.5%)

Table 6: Cell viability of A549 cells was tested after infection with *A. fumigatus* spores in relation to not infected cells incubated under the same conditions (negative control). Infected cells showed lower viability than not infected cells. Infection with Δ gntA (conducted in exp. 5, 13, 14, 15) mutants caused higher viability rate than in wildtype infection.

A. Dose-response



B. Cell survival after infection with Δ gntA spores

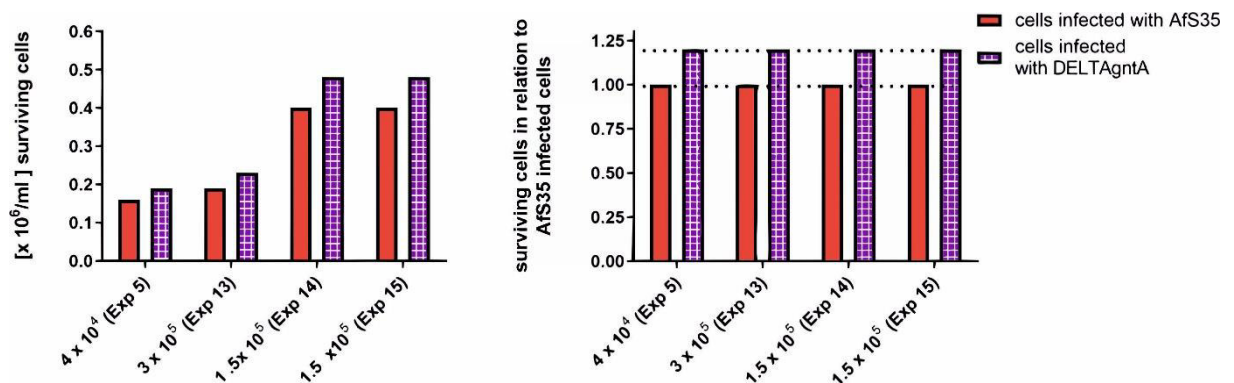


Figure 18: The graphs show the cell survival rates after A549 infection with *A. fumigatus* spores. A. In experiments with Afs35 spore infection we detected a linear dose-response relation. Increasing spore concentrations lead to decreasing cell survival. Left: absolute cell number; right: relation in %. B. In experiments with Af-3c lacking mutant Δ gntA we observed an increased number of surviving cells compared to the wildtype infected cells which was reproducible for different spore concentrations.

Interestingly, cells infected with Δ gntA spores show a higher survival rate than wildtype infected cells indicative of lower cell damage (see figure 18). In each experiment, the ratio of viable cells after infection with Afs35 and Δ gntA was steady ($[Afs35/\Delta gntA] = 0.833$) (table 7). This suggests that Af-3c plays a role in cell damage and, thus, also in virulence.

Experiment number	Spore concentration per sample	Surviving cells after infection with AfS35 spores [x 10 ⁶ cells/mL]	Surviving cells after infection with ΔgntA spores [x 10 ⁶ cells/mL]	Ratio [AfS35/ ΔgntA] infection
5	4x10 ⁴ spores/mL	0.16 (79%)	0.19 (95.3%)	0.16/0.19 = 0.84
13	3 x 10 ⁵ spores/flask (3 mL)	0.19 (12.7%)	0.23 (15.7%)	0.19/0.23 = 0.83
14	1.5 x 10 ⁵ spores/flask (3 mL)	0.4 (25%)	0.48 (30%)	0.4/0.48 = 0.83
15	1.5 x 10 ⁵ spores/flask (3 mL)	0.4 (24.5%)	0.48 (29.4%)	0.4/0.48 = 0.83

Table 7: The experiments 5, 13, 14, 15 compared infection outcome with AfS35 and ΔgntA spores. We observed a steady survival ratio of wildtype in relation to mutant infected cells (0.833). In relation to wildtype infected cells as reference, we observe a steady higher survival $1/0.83 = 1.2$.

Also, the surviving cell ratios, when we compare infection with different spore concentrations, show consistent results for both strains and correlate with the spore concentration ratio (table 8).

Experiment number	Spore concentration per sample		Surviving cells after infection with Af35 spores [x 10 ⁶ cells/mL]	Surviving cells after infection with ΔgntA spores [x 10 ⁶ cells/mL]
13	3 x 10 ⁵ spores/flask (3 mL)		0.19 (12.7%)	0.23 (15.7%)
14	1.5 x 10 ⁵ spores/flask (3 mL)		0.4 (25%)	0.48 (30%)
15	1.5 x 10 ⁵ spores/flask (3 mL)		0.4 (24.5%)	0.48 (29.5%)
Ratio of spore conc. between exp 13 and 14 respectively 15	3/1.5 = 2	Ratio of surviving cells between exp 13 and 14 respectively 15	0.19/0.4 = 0.48	0.23/0.48 = 0.48

Table 8: Ratio of surviving cells in infection with different spore concentrations shows steady results for wildtype and mutant strain. Double spore concentration causes half the cell survival rate; higher spore concentration causes higher cell damage. Cell damage seems to be a linear function of the spore concentration used for infection.

The figure below shows the percentage of surviving cells in uninfected condition and after infection with wildtype *Aspergillus* strain AfS35 and the mutant ΔgntA as shown in the experiments 13 to 15 (figure 19). The t-test reveals a reproducible impact of *Aspergillus* cell infection depending on the Af-3c production properties of spores.

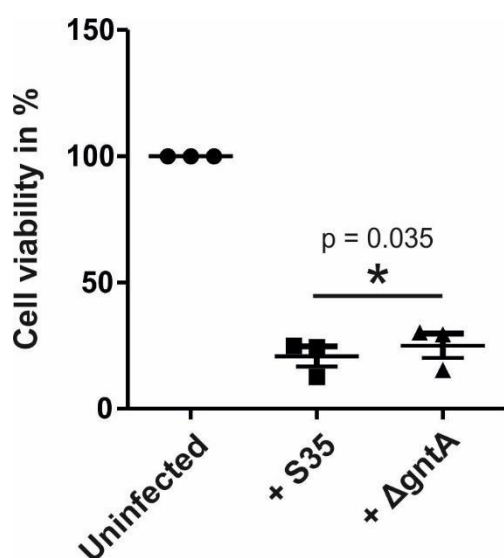


Figure 19: Data of experiment 13, 14, 15 of uninfected cells and cells infected with AfS35 (+ S35) and ΔgntA (+ ΔgntA). The result shows a statistically significant distribution of the values of cell viability after infection around the middle value with $p < 0.05$.

4. DISCUSSION

Our investigation focused on the biosynthesis pathway of the unusual zwitterionic glycolipid Af-3c in the opportunistic fungus *A. fumigatus* and its role in infection. This glycolipid containing an unusual GlcN- α -(1,2)-IPC core structure belongs to the glycoinositolphosphoceramides (GIPC) but in contrast to acidic GIPCs with a mannose core, Af-3c disposes both of an acidic phosphate and of a basic glucosamine, which grants its zwitterionic character (26,40). GIPC form a subcategory of glycosphingolipids (GSL), a major eukaryotic membrane component. However, since they are not present in mammals, GIPCs or the respective glycosyltransferases could be selectively targeted to develop antifungals (40).

Af-3c is the most abundant GIPC produced in *A. fumigatus* (26,37) and has also been described in the pathogenic fungus *Sporothrix schenckii* (41). Also the phytopathogens *Acremonium spp.* and *Trichoderma viride* synthesise similar GIPCs with a GlcN- α -(1,2)-IPC core (25,42). Previous experiments in the research group suggested that Af-3c is encoded by a gene cluster containing three glycosyltransferases and one de-N-acetylase (see figure 20) acting on IPC (29). The majority of gene clusters encodes fungal secondary metabolites such as the well-known antibiotics penicillin and cephalosporin, the immunosuppressant cyclosporin or cholesterol lowering drug lovastatin, to only cite a few (43,44). In *A. fumigatus*, the virulence factor galactosaminogalactan is a rare example of primary metabolite encoded by a gene cluster (45,46). The organisation of the Af-3c encoding genes in a cluster suggest that this glycolipid provides an advantage to *A. fumigatus* in competitive environments such as the soil or the lung, where it has to compete with bacteria. We are thus particularly interested in understanding the function of this peculiar glycolipid.

Though Af-3c could be produced in yeast by recombinant expression of the four identified biosynthetic enzymes, the amount obtained in this model was low (unpublished result). This low yield is likely due to an insufficient import of UDP-GlcNAc into the Golgi apparatus. Indeed, GntA, the enzyme involved in first step of Af-3c biosynthesis acts in the Golgi lumen and requires UDP-GlcNAc as donor substrate (29). A nucleotide sugar transporter (NST) with specificity for UDP-GlcNAc is thus necessary to transport UDP-GlcNAc from the cytosol to the Golgi for Af-3c biosynthesis. In contrast, other currently known fungal glycans synthesised in the secretory pathway such as N- or O-glycans do not contain any N-acetylglucosamine (GlcNAc) and import of UDP-GlcNAc in the organelles is not required for their biosynthesis (47). NSTs belong to the solute carrier 35 family (SLC35) and form a highly conserved family of type III transmembrane proteins that can be identified by BLAST searches. Their function is highly conserved from eukaryotes, fungi and parasites to mammals (47). Interestingly, a gene encoding a potential NST is located immediately downstream of the gene encoding GntA in *A. fumigatus*. In this work, we performed first steps towards the characterisation of this putative NST and provided first evidence of its involvement in UDP-GlcNAc import consistent with a role in Af-3c biosynthesis. We named this NST, UGT for **UDP-GlcNAc transporter**. We furthermore demonstrated that Af-3c is involved in damage to host cells suggesting that it plays a role in the fungus' virulence.

Glycoinositolphosphoceramides: GIPCs

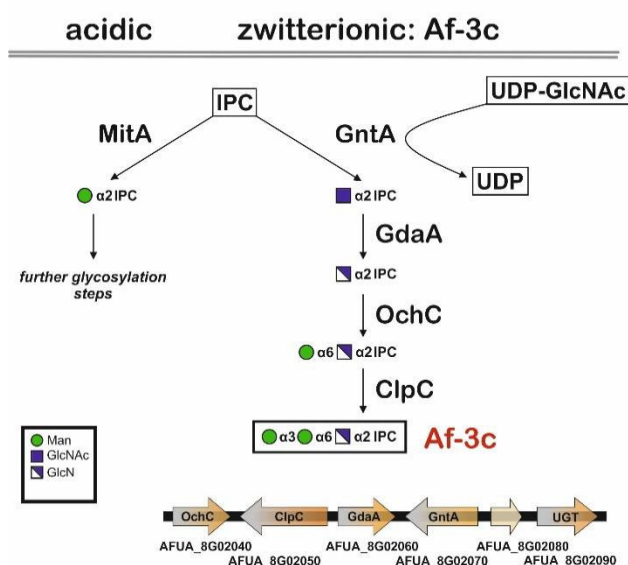


Figure 20: Af-3c represents a zwitterionic GIPC which contains both an acidic phosphate and a basic glucosamine. Substrate for the synthesis is UDP-GlcNAc, the gene cluster that is responsible for Af-3c synthesis has been identified in the past and encodes for the enzymes GntA, GdaA, OchC, ClpC.

NSTs typically contain ten transmembrane domains (48). The purification and crystallisation of two of NSTs, namely a GDP-mannose transporter and CMP-sialic acid transporter, only succeeded in recent years and provided first elements for understanding nucleotide sugar recognition (49,50). Reconstitution of the purified transporter into liposomes demonstrated that the lipid composition of the membrane is crucial for the transport function (50). Because of these characteristics, methods to study NSTs (or transporters more generally) are still very limited. The method of choice to analyse the specificity of NSTs relies on their expression in the yeast *S. cerevisiae*, which represents a low background system. Indeed, microsomal fractions isolated from yeast have been shown to transport GDP-mannose and UDP-glucose whereas transport of other nucleotide sugars is negligible. A microsomal fraction was isolated from the yeast expressing the NST of interest and its ability to import various radioactively labelled nucleotide sugars was measured (figure 6) as it was shown, for example, by Ashikov and colleagues or by Li et al. (32,51).

As in previous studies, we chose to express the putative UGT in *S. cerevisiae* using a vector containing a copper inducible promoter and enabling fusion with a N-terminal FLAG tag to assess the level of protein expression (32,51). The cloned cDNA encoded a putative protein with high conservation with other NSTs and ten predicted transmembrane domains, typical for this class of molecules (48) and showed some differences with the database predicted sequence. The plasmid encoding FLAG tagged UGT was successfully transformed into yeast and expression of the protein was induced. Unfortunately, the numerous antibodies tested against the FLAG tag epitope, all presented a high unspecific labelling (background) on Western blot, which precluded ascertaining the level of expression of the transporters. To solve this problem, we modified the vector containing the copper inducible promoter to enable addition of a N-terminal V5-tag to proteins and prepared new plasmids encoding V5 tagged UGT as well as the characterised human UDP-GlcNAc transporter SLC35A3 as control (52). The plasmids were transformed in *S. cerevisiae* and protein expression was induced with copper sulfate for two hours. Western blot analysis of Golgi enriched fractions showed expression of UGT and thus supported a Golgi location, as expected. Indeed, the UDP-GlcNAc transferase GntA has been shown to act in the Golgi apparatus of *A. fumigatus* (29).

Expression of the transporter was low but clearly visible. A high expression level is not desired since an overload of transmembrane proteins disrupt the Golgi apparatus integrity and lead to the obtaining of fragile vesicles unsuitable for *in vitro* transport assay.

In vitro transport assays were carried out with microsomal preparations and radioactive nucleotide sugars. Endogenous UDP-Glc import was first used as a control for the sealing of the isolated vesicles. Isolation of a suitable Golgi-rich fraction is indeed a delicate procedure that relies on a mild lysis of spheroplasts. The assays gave first indication that UGT is in fact a UDP-GlcNAc transporter. Difficulties in confirming this initial result could be due to the low expression of the transporter. But induction with cupric sulfate at higher concentrations bears toxic potential and the risk of cell damage. Degradation of the radioactive UDP-GlcNAc stock might also account for the low transport measured. NSTs are indeed antiporters that import a nucleotide-sugar into the organelles and export the corresponding nucleotide monophosphate in the cytoplasm but can also act as a simple mobile carrier (47,53). In consequence, degradation of the radioactive stock in GlcNAc, UDP and UMP would not only restrict availability of the substrate but UMP would also competitively inhibit transport of UDP-GlcNAc. The experiments should therefore be repeated with a new batch of radioactive UDP-GlcNAc solution to confirm our initial result.

In a follow up project, Isabel Ramon Roth in the group of Prof. Routier deleted the gene encoding UGT and demonstrated that this deletion resulted in complete absence of Af-3c and an increase of the precursor IPC (Master thesis of Isabel Roth, in preparation). This result demonstrates the involvement of UGT in the first step of Af-3c biosynthesis (transfer of GlcNAc to IPC) and thus supports its role in UDP-GlcNAc transport.

NSTs show high sequence identity with each other but the conservation is a poor indication of the specificity, which has to be determined experimentally. It is thus impossible to identify UGT in other fungi based on a simple BLAST. Similarly, the glycosyltransferases GntA, OchC and ClpC involved in Af-3c biosynthesis present several homologues in fungal species (54). Also the GlcNAc de-N-acetylase GdaA is homologous to the PIG-L/GPI12 deacetylase involved in glycosylphosphatidylinositol (GPI-) anchor biosynthesis (55). Using a combination of BLAST searches and cluster analysis, we identified a potential Af-3c cluster containing homologues of the three glycosyltransferases GntA, OchC and ClpC, the deacetylase GdaA and the UDP-GlcNAc transporter in *A. niger* and *A. clavatus* (figure 8). Future analyses will define if the genes are expressed and Af-3c is synthesised in these filamentous fungi. Interestingly, both *A. niger* and *A. clavatus* may cause invasive aspergillosis but much less frequently than *A. fumigatus*. In the case of the cluster encoded polysaccharide galactosaminogalactan, it was shown that increasing the level of this virulence factor in *A. niger* increased adhesion and virulence of the fungus (56).

The restricted taxonomic distribution of the genes and their organisation in cluster suggest horizontal gene transfer. Such transfer is particularly frequent in filamentous fungi and often confer an adaptive advantage to the host organism (44). Many of the functions gained support survival and adaptation to fluctuating environments (57). Deficiency of Af-3c is not detrimental to growth of *Aspergillus fumigatus* *in vitro* (29). Moreover, upregulation of the Af-3c clustered genes was observed when *A. fumigatus* was grown in aerial static conditions – conditions that mimic growth in the lung and are associated with reduced sensitivity to antifungal drugs (11).

Since *Aspergillus fumigatus* is an airborne pathogen, co-cultivation of strains provides a risk of contamination. In order to perform functional experiments, we first carefully checked the different strains for absence of contaminations. This was carried out by checking the antibiotic resistance of

the different strains and ascertaining the absence or presence of Af-3c by mass spectrometry. In conformity with our expectations, HPTLC and mass spectrometry showed different glycolipid profiles between the *A. fumigatus* strains. However, the reconstituted mutant $\Delta gntA$ -reintro showed lower Af-3c level than wildtype *A. fumigatus*. Reintroduction of the *gntA* gene in the deletion mutant involved the insertion of a rather large cassette with promoter, terminator and antibiotic resistant gene (29). The genomic organisation of Af-3c biosynthetic genes in cluster and very short space between the genes suggest that the genes are co-transcribed. Co-expression of gene clusters is often observed in eukaryotes and has been shown to reduce fluctuations in the levels of different components acting in concert in a specific pathway or forming a protein complex (58). Here the introduction of the cassette might disrupt the stoichiometry of the enzymes and/or expression of the UDP-GlcNAc transporter UGT and lead to a lower expression of Af-3c. The reconstituted mutant is therefore not an ideal control. Recent progress with CRISPR CAS technology in filamentous fungi enables more precise genomic manipulation (59). In the future, the *GntA* or UGT gene should be deleted and reintroduced without disturbing the cluster organisation.

The potential presence of Af-3c in spores was also investigated. Our experiment failed to detect Af-3c in spores. This is in agreement with Lambou et al. (54) who analysed the expression of several glycosyltransferases including OchC (named *AfOch3p*) without defining their functions. Quantitative PCR showed, that the α -(1,6)-mannosyltransferase OchC is not detectable in resting conidia but starts only with their swelling (54). Thus in the following experiments that use spores, Af-3c will be present after swelling of the spores and after formation of germ tubes, which takes approximately 6 to 8 hours.

To analyse the importance of Af-3c in *A. fumigatus* virulence, the Af-3c deficient mutant $\Delta gntA$ could be tested in a murine model of aspergillosis. However, in order to reduce animal experiments, methods to predict the implication of specific metabolites in *A. fumigatus* virulence are needed. Here, we wished to develop a simple assay to define the cellular damage created by a specific *A. fumigatus* strains and apply it to the Af-3c deficient mutant $\Delta gntA$. Indeed damage of pulmonary epithelial cells by *A. fumigatus in vitro* has been shown to correlate with virulence in mouse models of invasive aspergillosis (60,61). However, the described method requires the labelling of cells with ^{51}Cr . To avoid the use of radioactivity, we established a method to assess the direct physical damage of cells by estimating cell detachment.

Several trials with various multiplicity of infection (MOI) and incubation time enabled us to establish a reproducible protocol. Infection of monolayer of A549 cells in T25 flask with 10^5 spores led to ~50% cell damage overnight, which would allow the identification of less or more virulent strains. It is established that approximately 30% of adherent conidia are endocytosed by A549 cells and killed in the acidic phagosomes after 24 h. The rest germinate externally, penetrate and damage the cells (62). Cell damage is thus proportional to the initial dose of infection as observed in our study.

Importantly, experiments comparing the Af-3c deficient strain $\Delta gntA$ and mother strain AfS35 indicated that the strain lacking Af-3c created less cell damage suggesting that Af-3c plays a role in *A. fumigatus* virulence. Future experiments are needed to confirm this result and analyse a reconstituted strain expressing similar Af-3c level as the wild type strain. The protocol established in this study could be applied to estimate the virulence of other *A. fumigatus* strains.

4.1 Outlook

In future work, the plasmids generated in this study can be used for reconstitution of the Af-3c biosynthetic pathway in yeast. Once sufficient yield of this untypical glycolipid is provided, Af-3c analysis could include carbohydrate-linkage characterisation by digestion with the known exo-hydrolases α -(1,6)-mannosidase, α -(1,2)/ α -(1,3)-mannosidase (both from *Xanthomonas manihotis*) and α -(1,2)-mannosidase (*Trichoderma reesei*) followed by chromatographic or mass spectrometric analysis to confirm the exact specificity of the glycosyltransferases involved (63).

Especially of interest is the question which function does Af-3c hold in the pathomechanism in a successful *A. fumigatus* infection in mammal cells. In this study we provided a basic cell infection method for the evaluation of *A. fumigatus* virulence that could be extended to other fungal strains. This method suggest that Af-3c plays a role in cell damage and thus in infection. This result can be confirmed using established infection model such as larvae of the wax moth *Galleria mellonella* and mice (64). These experiments will require the prior reconstitution of the gene cluster to restore an Af-3c level similar to wild type *A. fumigatus*.

Glycans often play a role in innate immunity through recognition by diverse pattern recognition receptors (PRRs) that lead to the secretion of pro- and anti-inflammatory cytokines. Af-3c can be extracted from the Δ MitA strain and interactions with C-type lectins could be investigated. The macrophage surface receptor monocyte inducible Ca^{2+} -dependent lectin CLEC4E (Mincle) is of particular interest since it has been shown to convey a potent immune reaction to bacterial glycolipids (65). Furthermore cytokine responses to the Af-3c glycolipid could be assessed with human precision cut lung slices (66). In her master thesis work, Carla Seegers established an *A. fumigatus* infection protocol for precision-cut human lung sliced which allowed the immune response measurement IL-8, IL-6, IL-1 β that is to consider for future Af-3c experiments (67).

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A. MALDI-TOF MS spectrum showing the glycolipid profile of wildtype AfS35 extracted from mycelium by homogenisation, centrifugation, saponification, BuOH extraction, C₁₈ clean-up and drying under N₂ stream: MIPC precursor Hex₂-IPC production at high levels, overlapping mass peak patterns of Hex₂-GlcN-IPC (Af-3c) and Hex₃-IPC at high levels. p.53

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Figure 20: Af-3c represents a zwitterionic GIPC which contains both an acidic phosphate and a basic glucosamine. Substrate for the synthesis is UDP-GlcNAc, the gene cluster that is responsible for Af-3c synthesis has been identified in the past and encodes for the enzymes GntA, GdaA, OchC, ClpC. p.62

7. ABBREVIATIONS

<i>A. fumigatus</i>	<i>Aspergillus fumigatus</i>
ABPA	allergic bronchopulmonary aspergillosis
Af-3c	unusual zwitterionic glycolipid made of Man- α -(1,3)-Man- α -(1,6)-GlcN- α -(1,2)-IPC
CCPA	chronic cavitary pulmonary aspergillosis
CFPA	chronic fibrotic pulmonary aspergillosis
CNS	central nervous system
CPA	chronic pulmonary aspergillosis
GIPC	glycoinositolphosphoceramide
GPI	glycosylphosphatidylinositol
GSL	glycosphingolipid
Hex	hexose
IA	invasive aspergillosis
IPC	inositolphosphoceramide
MCS	multiple cloning site
MIPC	mannosylinositolphosphoceramide
NS	nucleotide sugar
NST	nucleotide sugar transporter
SAIA	subacute-invasive aspergillosis
TMD	transmembrane domain
UDP-Glc	uridine diphosphate glucose
UDP-GlcNAc	uridine diphosphate N-acetylglucosamine
UGT	UDG-GlcNAc transporter

9. DECLARATION ACCORDING TO: § 6 Abs. 2 Nr. 7 PromO (dt.)

Erklärung nach § 2 Abs. 2 Nr. 7 + 8

Ich erkläre, dass ich die der Medizinischen Hochschule Hannover zur Promotion eingereichte Dissertation mit dem Titel ***Biosynthesis and Function of Aspergillus fumigatus Zwitterionic Glycolipid (Af-3c)*** im Institut für klinische Biochemie (MHH) unter Betreuung von Prof. Françoise Routier in Zusammenarbeit mit dem Fraunhofer Institut für Toxikologie und experimentelle Medizin (ITEM Hannover) ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Die Gelegenheit zum vorliegenden Promotionsverfahren ist mir nicht kommerziell vermittelt worden.

Insbesondere habe ich keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Weiterhin versichere ich, dass ich den beantragten Titel bisher noch nicht erworben habe.

Ergebnisse der Dissertation wurden in keinem Publikationsorgan veröffentlicht.

Hannover, den _____

Patrizia Cardillo

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