RNA as a mediator of host-fungal pathogenesis

Alexander Bruch, Matthew G. Blango

Leibniz Institute for Natural Product Research and Infection Biology–Hans Knöll Institute (Leibniz-HKI), Adolf-Reichwein-Str. 23, 07745 Jena, Germany.

Email: alexander.bruch@leibniz-hki.de
Email: matthew.blango@leibniz-hki.de

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Contents

I. Abstract

In this chapter, we explore the wide range of functions RNA plays in mediating interactions between fungi and their hosts in the animal kingdom. We cover examples of RNA in regulation of gene expression, novel RNA structures and non-coding RNAs with links to fungal biology, as well as the emerging fields of epimutation and extracellular RNA. Next, the host response to fungal infection is investigated by considering cases of RNA recognition by host pattern recognition receptors, together with host regulatory mechanisms dependent on non-coding RNAs. Many of these themes are then revisited in the discussion of interkingdom communication, where RNA is sent between fungi and animals to influence the outcome of a given infection. We end by discussing the recent progress in RNA-based therapeutics against human fungal pathogens, where this foundation of basic research is leading to potential improvements in the way we treat human diseases.

II. Introduction

When discovered, nucleic acids were first associated with the source of their isolation. What was initially known as “thymus nucleic acid”, would eventually be defined as a specific macromolecule, deoxyribonucleic acid (DNA); the mysterious “yeast nucleic acid” was in due course classified as ribonucleic acid (RNA). In this chapter we will focus on the yeast nucleic acids and explore the contribution of the RNA macromolecule to fungal-animal relationships, with a particular emphasis on human fungal pathogens. We will examine far-reaching examples of RNA regulation in a variety of systems and explore concepts that will likely prove key in host-fungal interactions as this field further develops. In the conclusion section, we will provide an update on recent progress in using RNA more widely in therapeutic intervention, both as a target and as a therapeutic itself.

Before revealing the complexity of RNA regulatory pathways in the host-fungal interaction, we first must know a bit more about RNA biochemistry. The core structure of the RNA macromolecule is composed of alternating phosphate groups and ribose sugars. The ribose sugars of RNA carry, attached at their 1’ position, the nitrogenous bases adenine (A), guanine (G), cytosine (C), and uracil (U)—notably not thymine (T) as in DNA—and a hydroxyl group at the 2’ position, which helps distinguish RNA from DNA (**Fig. 1**). Unlike DNA, RNA is primarily found to be single-stranded, but this does not mean the molecule is wholly unstructured. In fact, the flexibility of the molecule and the incorporation of >170 modifications (Frye et al., 2018, Zhang et al., 2023) means that RNA is frequently folded into complicated secondary, and even tertiary structures (Väre et al., 2017). The structure of the tRNA is often used to illustrate the importance of RNA folding, with its numerous hairpins forming into a cloverleaf structure that finally twists into a more compact L-shaped structure via tertiary interactions between the helices.

RNA nucleotides can base pair in a manner similar to DNA, with guanine pairing with cytosine, and adenine pairing with uracil according to the standard Watson and Crick base pairing rules. One of the features that makes RNA so special is the ability of the bases to also pair in unconventional ways using the Hoogsteen edge or even the sugar edge for instance. Modified bases like pseudouridine or inosine inject even more possibilities into RNA folding and are likely only the tip of the iceberg of RNA folding complexity found throughout the kingdoms of life. Recent efforts in modeling RNA folding have revealed a highly dynamic process that can be facilitated by binding to proteins or other RNA molecules to form intermolecular structures (Yu et al., 2021).

With the discovery of messenger RNA (mRNA) in the early 1960s, RNA was divided into coding and non-coding sequences. In eukaryotes, RNA polymerase II typically transcribes mRNA, a process that is coupled with splicing, polyadenylation, and the addition of a 5’ protective cap structure. A combination of proteins and non-coding RNAs including transfer RNAs (tRNA) and ribosomal RNAs (rRNA) then help to decode and translate the mRNA into protein. In subsequent years, numerous additional regulatory RNAs have been described, including long non-coding RNAs (lncRNA) and a variety of subclasses of small RNA molecules like the microRNAs (miRNA) and short interfering RNAs (siRNA) that will be discussed further in this chapter. Collectively, these non-coding RNA species modulate gene expression by aiding in RNA processing, modification, and even trafficking.

The integral nature of RNA throughout gene expression and regulation means that it is also frequently involved in host pathogenesis, both as a modulator of the immune response and as a signal of foreign entities. In looking at the case of human-viral interactions, RNA intermediates like double-stranded RNA (dsRNA) are frequently recognized as a signal of a viral infection by pattern recognition receptors. In bacteria, small RNA species aid in virulence and gene regulation during infection, whereas many hosts send opposing signals as RNA molecules to promote immunity. We

have learned time and again that RNA is involved in signaling between organisms and can even play important roles in immune recognition pathways.

The most notable examples of intercellular RNA communication come from plant-fungal interactions, where studies of bidirectional cross-kingdom RNA interference have revealed that small RNAs are produced by both the host and the pathogen to influence the outcome of infection in a sort of molecular arms race (Weiberg et al., 2013, Cai et al., 2018b). Canonically, these small RNAs are produced by dsRNA-precursor cleavage by a dicer protein before loading into an argonaute protein for gene silencing, often as part of a larger RNA-induced silencing complex (Wilson and Doudna, 2013). Sequence complementarity between the small RNA and target drives mRNA degradation or translational repression. As an example, the small RNAs of the grey mold *Botrytis cinerea* were shown to be transferred to the plant host and internalized via clathrin-mediated endocytosis to silence plant immunity genes (Weiberg et al., 2013, He et al., 2023). The host plant also uses a similar approach to fight back against fungal infection by delivering effector small RNAs and mRNAs in exosome-like extracellular vesicles into the fungus (Cai et al., 2018b, Wang et al., 2024). The endogenous small RNAs produced by plants like *A. thaliana* are transferred to the fungus to target the mRNAs of conserved vesicle pathways important to virulence (Cai et al., 2018b, He et al., 2021). Other examples support the use of small RNAs by plants to target fungal pathogens as well. Wheat plants can suppress the invasion of *Fusarium graminearum* using miRNAs (Jiao and Peng, 2018), and cotton plants export miRNAs to inhibit the virulence of *Verticillium dahliae* (Zhang et al., 2016). These studies offer a clear role for RNA in interkingdom communication in plant-fungal interactions.

In this chapter, we extend these principles to explore the wide range of functions RNA plays in interactions between fungi and their hosts in the kingdom *Animalia*. Most of these cases come from studies of human fungal pathogens due to their medical relevance, but we expect that these mechanisms are by no means unique to pathogens. Instead, similar processes are surely used during all sorts of host-microbe interactions. The studies presented here will highlight how much we can learn by studying RNA regulation in pathogens and how animal hosts naturally respond to these fungal invaders. We will end by discussing the potential of converting our collective knowledge of RNA regulation in fungal pathogenesis into RNA centric therapeutics to fight back against fungal pathogens and improve human health.

III. RNA Regulation in Fungal Pathogens

A. RNA in gene regulation: transcription and translation

The classic, textbook function of RNA is in converting the genetic information of DNA into the functional output of protein, but we now know that RNA serves many more roles beyond just that of an intermediary. In the process of transcription, RNA serves as both the product of the RNA polymerase and as a regulator of the entire process of transcription in the form of non-coding RNAs. We have learned a lot from model organisms about the intricacies of RNA regulation of transcription, but in many cases, we have obtained only glimpses of the complexity surrounding RNA regulation in fungal pathogens. The transcriptional response as assessed by RNA-seq is now frequently the first feature of gene expression considered when investigating a new experimental system.

Cryptococcus neoformans is likely the best-studied of the human pathogenic fungi regarding central questions surrounding gene regulation. It was recently shown that upon entry of *C. neoformans* into the host environment, many gene regulatory systems are employed to facilitate global gene expression reprogramming and adaptation to the local host environment. A major aspect of this adaptation comes in the form of temperature response to adjust to the warm host, an essential attribute of any human fungal pathogen. For *C. neoformans*, it was shown that the mRNA

decay pathway plays a pivotal role in managing thermotolerance and growth in the host by degrading messages, in particular those of ribosomal proteins (Bloom et al., 2019). In the absence of the mRNA decay pathway using a *ccr4* knockout strain, cell wall remodeling enzymes were repressed, resulting in exposed cell wall glucans on the fungal surface that could trigger activation of the C-type lectin receptor Dectin-1. Recognition of fungal ligands by Dectin-1 led to an increased host response and clearance of the fungus, highlighting the importance of removing unwanted transcripts to facilitate a more fine-tuned transcriptomic response.

The rapid adjustment of transcriptomes during the interaction of organisms is facilitated by global regulatory proteins like the *C. neoformans* virulence-associated DEAD-box RNA helicase VAD1. This RNA binding protein functions as a negative regulator of the global transcriptional repressor, NOT1, resulting in upregulation of a laccase important for melanin pigment production during infection (Panepinto et al., 2005). Corroborating that the loss of VAD1 was detrimental to virulence, other virulence determinants, including TUF1, PCK1, and MPF3 were also downregulated. Global regulators like VAD1 play important roles in rapidly shaping the transcriptome during the host-pathogen interaction, where integration of virulence cascades into the global transcriptional response is paramount.

Yet, it is not always the case that pathogens respond so aggressively to the new host environment. In fact, the filamentous fungal pathogen *Aspergillus fumigatus* does not mount a recognizable transcriptional response to human airway epithelial cells at all (Watkins et al., 2018), at least in culture, but instead responds more actively to the growth media of these *in vitro* assays. These findings suggest that understanding the entire host environment, including cells, nutrients, and likely otherwise unanticipated features will be important to eventually appreciate gene regulatory cascades and transcriptional responses employed during interaction.

Once mRNA is produced, it must be translated by the ribosome into protein; a process influenced on multiple levels by features of the mRNA, additional non-coding RNAs, and even catalytic RNA molecules in the supramolecular structure of the ribosome. The ribosome is a dominant feature of life that may superficially seem extremely conserved, yet examples of alternative regulation exist even here. One such story comes from the yeast *Candida albicans*, an organism capable of living as both a commensal and a pathogen in human hosts. Unlike the model yeast *Saccharomyces cerevisiae, C. albicans* employs a ribosomal RNA processing cascade that uses both cotranscriptional and post transcriptional rRNA processing modes to facilitate better responses to changing environmental conditions like those found in a nutrient restricted host (Pendrak and Roberts, 2011). By evolving this layered regulatory network, *C. albicans* has acquired an additional tool to manage stressful situations and survive in a harsh host environment. This finding represents the power of studying non-model organisms to reveal amazing new regulatory mechanisms of core molecular processes.

Translation is greatly influenced by the architecture of the mRNA, including parameters such as length, structure, and regulatory elements. In *Cryptococcus*, mRNAs have long transcriptional leader sequences and appear to employ a large number of upstream open reading frames (uORFs) capable of repressing translation, features that are less prevalent in *S. cerevisiae* (Wallace et al., 2020). The observed uORF repression has a strong dependency on the Kozak sequence context of the associated AUG start codon, and a strong context typically promotes nonsense-mediated decay of these mRNAs. It was also shown that start codon context contributes to regulation of predicted dual-localized proteins, a mechanism that is hypothesized to occur as a leaky upstream AUG is separated from a downstream in-frame AUG with strong Kozak context by a mitochondrial localization signal. The

selection of start codons thus dictates the abundance and structure of the proteins produced; a characteristic that is now recognized to be maintained in many fungi across the kingdom.

Technological advances in RNA-seq have facilitated incredible progress in our understanding of gene expression, but in general the tools to study many aspects of gene regulation in fungal pathogens remain missing. In many cases the complex and impermeable cell wall that can scavenge nucleic acids from the environment has proven to be a significant hurdle (Jochl et al., 2009). Molecular and cellular techniques are improving with time and studies that were difficult a decade ago are now becoming more common. For example, mRNA localization studies were nearly impossible a few decades ago, as high background and low signal made data interpretation difficult. Early experiments were promising, and despite these technical challenges, indicated a regulated trafficking of mRNAs in fungi, specifically in organisms like *C. albicans*, where mRNAs were shown to be trafficked to the hyphal tip to aid hyphal development and even invasion during infection (Elson et al., 2009). More recent advances in fluorescent *in situ* hybridization techniques like hybridization chain reaction using split probes have provided improved signal-to-noise ratios and allowed single molecule visualizations to work in some organisms (Moreno-Velasquez and Perez, 2021). In this case, multiple mRNAs were visualized together in a single *C. albicans* cell with minimal loss of signal, paving the way for more mechanistic studies of mRNA trafficking and regulation in this and related organisms. Improvements in localizing and tracking RNAs is now facilitating studies of the full lifecycle of a fungal RNA from transcription to degradation.

B. RNA Structure and non-coding RNA

In addition to new variations of canonical processes, research has uncovered a tremendous number of novel RNAs and RNA structural motifs in fungi. Large-scale screening of fungal genomes revealed an abundance of unique structural folds, some of which could be assigned functions (Li and Breaker, 2017). However, most of these RNA structural motifs and non-coding RNAs remain completely unstudied, leaving a wealth of untapped information available to the field. The study of both RNA (and DNA) G quadruplexes, noncanonical four-stranded structures formed by a series of G-G base pairs, is a growing area of research in many organisms, including fungi. Recent efforts have revealed widespread examples of these intriguing secondary structures in *C. neoformans* and *A. fumigatus* (Leipheimer et al., 2018, Warner et al., 2021), but the full regulatory potential remains unknown. Fungal riboswitches are rarer, with only a few variations of the thiamine pyrophosphate (TPP) riboswitch described to date (Vargas-Junior et al., 2022, Moldovan et al., 2018, Donovan et al., 2018). It can be guaranteed that novel structures and regulatory mechanisms will continue to be unearthed as the field expands into new transcriptomic territory.

In the genus *Candida*, a recent study uncovered thousands of lncRNAs that show evolutionary conservation above that observed for intergenic regions but less than protein-coding regions (Hovhannisyan and Gabaldon, 2021). These moderately conserved lncRNAs were also observed to be differentially expressed during infection of epithelial cells with *Candida* species, suggesting a potential function in regulation of virulence or stress response; however, more work will need to be done to elucidate the full impact of these lncRNAs on gene regulation. Corroborating the likely importance of these newly identified lncRNAs, the *C. auris* lncRNA *DINOR* was shown to contribute to stress response and virulence, with a genetic deletion exhibiting increased sensitivity to stressors and decreased virulence in a mouse model of infection (Gao et al., 2021). Work in *C. neoformans* has uncovered an estimated 1182 lncRNAs, with diverse predicted functions (Kalem and Panepinto, 2022). Concerted effort is now rightly underway to characterize these widespread regulatory RNAs.

The first lncRNA characterized from a human pathogen came from *C. neoformans* and can serve as an example of the regulatory potential waiting to be uncovered in lncRNAs. In this study, the lncRNA *RZE1* was shown to regulate the anti-virulence factor and morphogenesis regulator, Znf2 (Chacko et al., 2015). The lncRNA *RZE1* is restricted to its nucleus of origin, where it works primarily in cis to increase the transcript level of *ZNF2* and alter the ratio of cytoplasmic to nuclear transcripts in favor of the cytoplasm, as determined using deletion studies. In the absence of the zinc finger transcription factor Znf2, *C. neoformans* cells are locked in the more virulent yeast form. Consistent with this regulation, the loss of *RZE1* promoted virulence by decreasing *ZNF2* transcript levels, linking its expression to pathogenicity. Although just a few examples, lncRNAs have a vast potential to control gene regulation, stress response, and virulence in diverse ways during interaction with host organisms.

Non-coding RNAs come in many forms, from the lncRNAs mentioned above to tRNA, rRNAs, or even as introns. In the case of introns, examples are scattered throughout the literature of the complex regulation both in removal of introns from mRNAs by the splicing machinery, as well as the intricate regulatory pathways that result in intron retention or alternative splicing under different conditions. In *C. neoformans*, introns can be retained for gene regulation in a manner that seems to be independent of nonsense-mediated decay (Gonzalez-Hilarion et al., 2016). This is in stark contrast to other organisms where introns are thought to diversify and provide additional flexibility to the proteome (Chorev and Carmel, 2012). Introns are also known to help protect eukaryotic genomes against the genomic instability introduced by transcription (Bonnet et al., 2017). It is then not surprising that there is evidence to support differential regulation of alternative splicing during fungal stress and virulence (Sieber et al., 2018). The picture becomes even more convoluted when additional regulatory RNAs are added into the mix. A clear illustration comes from *A. flavus*, where natural antisense transcription, another form of poorly understood non-coding RNA, was found to be controlled in part by temperature; however, the full implications of this finding remain unknown (Smith et al., 2008). A more careful and systematic assessment of the many factors involved will likely be required to fully understand the regulatory potential of RNA structure and non-coding RNAs during interactions between fungi and animals.

C. RNAi and small RNAs: Regulation of resistance and genome defense

The best-studied RNA regulatory pathway in non-model fungi is likely the RNA interference system. Over the past two decades, many fungi have been shown to contain functional RNAi pathways, with *Cryptococcus* spp. and *A. fumigatus* among the first to be characterized (Janbon et al., 2010, Liu et al., 2002, Mouyna et al., 2004, Hammond et al., 2008). The canonical RNA interference system processes dsRNA to sequence-specifically, posttranscriptionally silence gene expression by mRNA cleavage or blockade of translation machinery binding (Wilson and Doudna, 2013). The RNAi machinery, and small RNAs generated by the system, can also promote the formation of a heterochromatic state, resulting in transcriptional repression (Martienssen and Moazed, 2015). In fungi, the RNAi pathway frequently plays an important role in genome defense from mycoviruses and selfish genetic elements like transposons (Priest et al., 2022, Navarro-Mendoza et al., 2023), but other varied functions have also been assigned (Torres-Martinez and Ruiz-Vazquez, 2017). However, functions are not widely conserved, with extreme variation between systems. Small RNA generation in fungi is therefore an active area of investigation, as few examples of canonical small RNA biogenesis pathways have been clearly defined (Jiang et al., 2012). Instead, small RNAs are generated by diverse pathways utilizing RNAi machinery often in conjunction with cellular enzymes that appear to have moonlighting functions in small RNA biogenesis (Lee et al., 2010).

Early on it became clear that fungi employ variations of the RNAi machinery during different growth phases, for example the sexual reproductive cycle or during vegetative growth. Pathways like quelling (Romano and Macino, 1992) or transgene-induced cosuppression (Wang et al., 2012) are active during vegetative growth, whereas meiotic silencing by unpaired DNA (MSUD)(Shiu et al., 2001), repeat-induced point mutation (RIP)(Gladyshev, 2017), and sex-induced silencing (Wang et al., 2010, Wang et al., 2013) reveal just a bit of the complexity that has been unearthed in the many variations of small RNA biogenesis during fungal sexual cycles. In asexual spores (conidia) of *A. fumigatus*, the RNAi pathway appears to play a role in supporting transcription of ribosome biogenesis genes (Kelani et al., 2023), a feature reminiscent of prior work proposing that RNAi is required for release of RNA polymerase in cycling and quiescent cells of *Schizosaccharomyces pombe* (Roche et al., 2016). More details on each of these pathways are unfortunately beyond the scope of this chapter.

The RNAi machinery also contributes to other noncanonical regulatory functions, features which were proposed to have accrued over multiple evolutionary gain and loss events (Bernstein et al., 2012, Billmyre et al., 2013). Remarkably, RNAi can even be lost completely by some organisms. Loss of RNAi in *C. gattii* leads to increased virulence and is thought to have been a factor for the success of the pathogen during the now famous pacific northwest outbreak of the 1990s (Datta et al., 2009). Many other members of the *Cryptococcus* complex have retained RNAi, again highlighting the countless paths to becoming a pathogen (Feretzaki et al., 2016). The loss of argonaute proteins in *C. gattii* does have tradeoffs though, as genome rearrangements and higher levels of retrotransposon expression and small RNA generation are observed (Ferrareze et al., 2017). The full consequences of RNAi loss in this organism is still under investigation, but these data support many other studies showing that the RNAi pathway is a major player in genome defense against selfish genetic elements. *C. gattii* also offers a system to explore novel small RNA pathways that are typically overshadowed by an intact, canonical RNAi system. One example of a dicer-independent pathway of transposon silencing has been described in *Cryptococcus* already (Burke et al., 2019). Instead of dicer, *Cryptococcus* uses *RDE3*, which encodes a protein with similarities to the *S. cerevisiae* RNase III protein Rnt1. Together with several other presumed nuclear factors, RDE3 can aid in production of endo-siRNAs and suppress transposon mobilization. Finally, RNAi has even been repurposed for entirely new functions. The reference strain of *C. albicans* uses dicer for optimal maturation of ribosomal and spliceosomal RNAs (Bernstein et al., 2012), replacing the functionality of the nuclear Rnt1 protein found in more ancestral strains.

Over the past decade, the role of RNAi in stress response and drug resistance has become more apparent with the description of RNAi-dependent epimutation. The majority of this work has been done in *Mucor circinelloides* and other closely related organisms (Calo et al., 2014), where unstable antifungal drug resistance has been attributed to RNAi-dependent epigenetic regulation. Several examples exist, but the best remains the *M. circinelloides* response to the antifungal drug FK506 (Chang et al., 2019, Calo et al., 2014). In response to challenge with FK506, *M. circinelloides* silences expression of the *fkbA* gene to limit production of the FKBP12 protein. The absence of FKPB12 prevents formation of a complex with FK506 that can inhibit the protein phosphatase calcineurin, resulting in resistance to FK506. The epimutation pathway requires several other peripheral RNAi factors, including the quelling induced protein (QIP) and a Sad-3-like helicase (RnhA) (Calo et al., 2017). These drug-resistant epimutants were found to be stable during infection in a mouse model, with clear clinical implications for application of antifungal therapy (Chang and Heitman, 2019). A noncanonical RdRP-dependent, dicer-independent silencing pathway reliant on R3B2 and RdRP3 was then shown to inhibit this RNAi-dependent epimutation pathway via mRNA degradation (Calo et al.,

2017). The concept is then that the RNAi-dependent epimutation pathway serves in stress response and is balanced by the mRNA degradation pathway that occurs during normal growth. Collectively, these findings highlight the interplay and repurposing of multiple RNAi components to create a balanced regulatory network in *Cryptococcus*. Future work will inevitably reveal how these, and other pathways have evolved in other fungal pathogens for the purposes of stress response and virulence.

In addition to microRNA-like RNAs (milRNAs) and siRNAs, other small RNA species are also found in fungi. *A. fumigatus* was among the first organisms shown to produce tRNA fragments under stressful environmental conditions, including starvation (Jochl et al., 2008). tRNA fragments are a heterogenous and diverse class of molecules typically produced from mature, full-length tRNAs by cleavage with enzymes like Rny1 or Dicer (Xie et al., 2020). tRNA fragments are known to have a variety of ambiguous functions, but conventional knowledge suggests cleavage of tRNAs frequently limits translation, with the tRNA fragments then capable of repressing gene expression. Despite the identification of tRNA fragments in *A. fumigatus*, little is known about the regulation and functional outcome of production of tRNA halves and smaller fragments in this organism. The world of small RNAs in fungi remains poorly described, and additional studies will certainly reveal new biology.

D. Extracellular RNA and extracellular vesicles

Decades of work have proven the importance of both coding and non-coding RNAs in regulation of nearly every process within the cell. In recent years, functions have also been emerging for RNA outside of cells. The extracellular RNA population has proven enigmatic, but advances in tools to study extracellular vesicles have revealed a more complex extracellular environment than previously appreciated. Studies in humans have revealed an extracellular RNA environment with RNA even taking on previously unrecognized folds, as observed for protected intermolecular interactions formed by some tRNA fragments (Tosar et al., 2020). Our understanding of extracellular RNA released by fungi has been driven by studies of plant-fungal interactions, as mentioned in the introduction (Weiberg et al., 2013). As early as 2009, evidence was emerging that fungal extracellular vesicles may contain RNA (Nicola et al., 2009). The use of membrane and nucleic acid dyes for the staining of extracellular fractions has proven challenging (de Voogt et al., 2021), but improved extracellular vesicle isolation protocols have facilitated new strategies. Together with the increased sensitivity of RNA-seq technologies, a number of studies have been performed confirming the release of extracellular RNA by fungi, including *C. albicans*, *C. neoformans*, *Histoplasma capsulatum*, and *Paracoccidioides brasiliensis* (Peres da Silva et al., 2015, Alves et al., 2019). Nearly all RNA species have been identified extracellularly, with small RNAs most frequently identified (Peres da Silva et al., 2015, Munhoz da Rocha et al., 2021, Rayner et al., 2017). Larger RNAs have also been detected, including mRNAs and lncRNAs in a variety of experimental systems (Peres da Silva et al., 2019) with a particularly noteworthy example of complete mRNAs being transported from plant host to fungal pathogen and translated (Wang et al., 2024).

How these RNA species traffic to the extracellular space remains unclear. The current consensus is that extracellular vesicles play a large role in carrying RNA and protecting it from the hostile extracellular environments. In *C. deneoformans*, the Cin1-Wsp1-Cdc42 endocytic pathway was proposed to play a role in RNA export by membrane vesicles, with deletion of *CIN1* resulting in major changes to the RNAs produced and released (Liu et al., 2020). In *C. neoformans* several factors are thought to contribute to RNA loading and export, including the Golgi reassembly and stacking protein GRASP (Peres da Silva et al., 2018) and the scramblase Aim25 (Reis et al., 2019). It is not yet clear if these preliminary discoveries are more widely applicable across fungi, but again, a lot of work is underway to answer these questions.

The functions of extracellular RNAs are just starting to be dissected. Most studies have again focused on plant-fungal pathogens, but some work has been done in human-fungal interactions too. Work from the important human pathogen *C. gattii* revealed it to use extracellular vesicles loaded with protein and RNA cargo to facilitate virulence (Bielska et al., 2018). During infection, the fungus uses a division of labor strategy driven by sensing of host reactive oxygen species, where a subpopulation of cells become dormant and facilitate the rapid growth of another subset of fungal cells (Voelz et al., 2014). This support is reliant on transfer of RNA and protein cargo by extracellular vesicles released from the fungus and taken up by host macrophages. Much remains to be discovered in this system, but it seems clear that fungi can use extracellular vesicles to their advantage to deliver cargo molecules like RNA between subpopulations in a host environment.

A particularly compelling example of the influence of extracellular RNA on host-fungal interactions comes from the insect pathogen *Beauveria bassiana*. During infection, *B. bassiana* releases a milRNA termed bba-milR1 that attenuates mosquito immunity by exploiting the insect RNAi machinery (Cui et al., 2019). The bba-milR1 milRNA was identified by searching for small RNAs produced during infection of mosquitos that were specific to the *B. bassiana* genome. Four small RNAs were identified using this dual RNA-seq approach. Bba-milR1 exhibited Dicer-dependent biogenesis via a Dicer-like 2 protein, and deletion of the milRNA led to increased survival of mosquito hosts during infection. RNA precipitation experiments with antibodies against the *Anopheles stephensi* AGO1 protein revealed binding of milR1-4 to the mosquito protein. These results suggested that *B. bassiana* milRs were exported into the mosquito to hijack the host RNAi machinery. Bba-milR1 shared complementarity with several mosquito immunity genes and was shown using dual luciferase reporter assays to regulate gene expression by decreasing expression of the Spätzle-like cytokine Spz4 and increasing expression of the CLIP serine protease CLIPB9. Antagonistic bba-milR1 agomirs blocked the gene regulatory effects, and *in vivo* studies confirmed that bba-milR1 is capable of silencing *spz4* during the cuticle penetration phase of the infection to limit antifungal activity of the mosquito and give the fungus an edge in the infection. In an interesting wrinkle, *B. bassiana* then lowers levels of bba-milR1 during hemocoel invasion late in infection to prevent activation of CLIPB9 and the protective host melanization response. Small RNA trafficking and regulation is tightly controlled during host-fungal pathogenesis, likely offering multiple ingress points for potential biocontrol strategies in the future.

E. Other examples of RNA regulation in fungi

There are too many studies of RNA regulation in fungi to cover each in depth here, but a few cases can provide evidence of the immense complexity yet to be revealed. The first example comes from a nontraditional RNase III enzyme found in *Mucorales*. Canonical RNase III enzymes cleave dsRNA to smaller precursor molecules that can contribute to gene silencing, as discussed above. However, recently a *Mucorales* RNase III enzyme was identified that evolved to cut single-stranded RNA (ssRNA) (Canovas-Marquez et al., 2021). This enzyme, known as R3B2, is an important regulator in the *Mucorales* noncanonical RNAi pathway mentioned above, which has been linked to regulation of virulence through degradation of specific mRNAs. In this system, the function of dicer is replaced with R3B2 and the system requires RNA-dependent RNA polymerase activity, but not argonaute proteins (Trieu et al., 2015), indicating an RNase III enzyme that displays a noncanonical cleavage profile. In addition to noncanonical roles in RNA cleavage, RNase orthologs also exist that completely lack RNase activity, as was shown for the RNase II enzyme Ssd1 in *S. cerevisiae* (Bayne et al., 2022). Ssd1 lacks RNase activity, but retains its ability to bind RNA, a feature which has been linked to its function of controlling stress response and virulence in a variety of fungi. A novel binding site on the

surface of the protein is thought to aid interaction, but it remains to be definitively proven whether the observations from model organisms like *S. cerevisiae* will extend to other fungi as well. Collectively, these studies provide a cautionary tale, highlighting potential difficulties in separating presumed enzymatic activity from unanticipated RNA binding functionalities.

The contribution of RNA modifications to regulation of gene expression during stress and host-microbe interactions is another emerging area of research. The field has largely focused on tRNA modifications, revealing that various important modifiers like Ncs2, the Elongator- and KEOPS complexes, as well as Sua5 contribute to the virulence of fungal pathogens like *C. albicans*, *A. fumigatus*, and *C. neoformans* (Zhang et al., 2022, Alings et al., 2023, Choi et al., 2023). Remarkably, all modifiers target the anticodon loop by either modifying wobble uridines (Ncs2, Elongator complex) or position 37 adenosines (KEOPS complex, Sua5), indicative of their importance for tRNA stability and decoding fidelity. In each case ablation results in decline of pathogenicity and stress resistance. In addition to tRNA modifications, additional work has linked modification of mRNAs to control of gene expression, intriguingly showing a requirement for m^6A methylation in biosynthesis of aflatoxin in *A. flavus* (Yang et al., 2023). A number of studies have shown similar roles for m^6A in many other fungal systems, implying a vast coordinated regulation in response to changing environmental conditions across fungi (Ren et al., 2022, Kim et al., 2023). Although still a field in its infancy, RNA modifications are likely to be revealed as key regulators of host-fungal interactions.

A final interesting regulatory feature of RNA comes in the form of RNA-directed modification. Many examples exist, but here we will focus on the tRNA-dependent addition of amino acids to glycerolipids. The addition of amino acids to glycerolipids is widespread in bacteria, but until recently no examples were described in fungi. Now we know that aspartate can be added to ergosterol in a tRNA-dependent fashion in a wide range of fungi, including important human pathogens like *A. fumigatus* (Yakobov et al., 2020). Ergosterol is the principal sterol in fungal cell membranes, and enzymes involved in ergosterol biosynthesis are frequent targets of antifungal therapeutics. It is therefore quite intriguing that the fungal enzyme ergosterol-3β-O-L-aspartate synthase (ErdS) transfers aspartate from Asp-$tRNA^{Asp}$ to the 3β-OH group of ergosterol. ErdS is a bifunctional protein that has an aspartyl tRNA synthetase fused to a Domain of Unknown Function 2156 domain that transfers the aspartate to the sterol. An additional enzyme ErdH is then capable of removing the added aspartate. A fuller description of the biosynthesis and regulation of ergosterol may reveal novel therapeutic targets, and certainly the tRNA-dependent addition of amino acids to glycerolipids represents just one of many fungal RNA modification reactions still hiding from view.

IV. Host RNA Responses

The human immune system, subdivided into the innate and adaptive immune response, must react and modulate its actions according to microbial threat. Recognition of fungal molecules serving as pathogen-associated molecular patterns (PAMPs) is mediated by pattern recognition receptors (PRR). Among these, the ten human Toll-like receptors (TLR) are a class capable of specifically binding different PAMPs to mediate innate immune reactions, leading to inflammation, immune cell recruitment, and phagocytosis of the respective microbe (Moresco et al., 2011). The first indications that these receptors were essential for antifungal defense came from a *Drosophila-A. fumigatus* infection model (Lemaitre et al., 1996), which were later supported by findings describing TLR2 and TLR4 as integral components in combatting fungal invaders like *C. albicans* and *A. fumigatus* (Bellocchio et al., 2004, Mambula et al., 2002, Meier et al., 2003, Netea et al., 2002). TLR2 binds to the cell wall component zymosan of *S. cerevisiae* with the help of TLR6 and also recognizes β-glucan in cooperation with Dectin-1 (Underhill et al., 1999, Gantner et al., 2003, Brown and Netea, 2007).

TLR4 is classically known to recognize bacterial lipopolysaccharides (LPS) but was also found to bind different mannan structures in *C. neoformans* and *C. albicans* to drive induction of proinflammatory responses (Netea et al., 2006, Tada et al., 2002, Shoham et al., 2001).

These examples illustrate that a set of receptors are capable of recognizing fungi by the molecules exposed on the cell wall, indicating that these receptors are located on the plasma membrane of the host cell. Interestingly, a variety of other receptors like TLR3, TLR7/8, TLR9, and the RIG-I-like receptors (RLRs) are found both in the cytoplasm (RLRs) and/or membrane associated, facing the endosomal/lysosomal lumen (TLRs) to recognize foreign nucleic acids (Uehata and Takeuchi, 2020). TLR3 and TLR7/8 specifically bind select RNA-species to activate the innate immune response (Jannuzzi et al., 2020). This section will discuss some of the recent data suggesting that PRRs can respond to RNAs associated with human fungal infection and contextualize these intriguing findings with other RNA-dependent regulatory systems activated upon host-fungal interactions in a range of organisms.

A. RNA recognition by Toll-like receptors in fungal immunity

Nucleic acids can be actively secreted by microbes within the host environment or released from dying or degraded microorganisms upon phagocytosis or endocytosis by host immune cells (Lee and Barton, 2014, Barton, 2007). TLR3 and TLR7/8 (as well as TLR9) are trafficked to either endosomes or lysosomes after synthesis in the endoplasmic reticulum, enabling the interaction and activation of these receptors by foreign nucleic acids (**Fig. 2**) (Lee and Barton, 2014, Barton, 2007). The RNA-specific TLRs appear to play a major role in the response to fungal infections by either recognizing dsRNA (TLR3) or ssRNA (TLR7/8) (Jannuzzi et al., 2020). This was demonstrated in a murine dendritic cell (DC) model system in which TLR3 was deleted and lung DCs were subjected to *A. fumigatus* infection (Carvalho et al., 2012). Lack of TLR3 affected the priming and MHC-I restricted protective memory response of $CD8^+$ T cells by DCs, which was caused by a decrease of the phagocytic index of conidia, no upregulation of *CCR7*, and finally reduced lung-to-lymph node migration. In essence, no recognition of fungal RNA by TLR3 led to an ablation of the critical *CCR7* induction strictly necessary for DCs to migrate to the lymph nodes and prime $CD8^+$ T cells. This higher susceptibility to *A. fumigatus* infection in mice was corroborated by a higher vulnerability of patients with a single nucleotide polymorphism (SNP) in the *TLR3* gene to develop aspergillosis (Carvalho et al., 2012). These patients exhibited defects in DC function and their capability to activate $CD8^+$ T cells.

Interestingly, recognition of pathogenic fungi by TLR3 is not limited to the detection of fungal RNA but can also be mediated by mycoviral dsRNA. Fungal pathogens from the genus *Malassezia*, known to commonly inhabit the human skin, were found to be infrequently infected by mycoviruses of the family *Totiviridae* (Saunte et al., 2020, Applen Clancey et al., 2020, Park et al., 2020). In general, the mycoviral genomes consisted of dsRNA segments of different sizes packed into viral particles but not excreted to infect other organisms. Instead, the transmission process relies on cell division, mating, or the fusion between individual vegetative cells (hyphal anastomosis) (Ghabrial, 2008, Ghabrial et al., 2015). An investigation focusing on *Malassezia restricta* identified a new mycovirus termed MrV40 that was extracted from clinical fungal isolates. The hallmark features of the MrV40-infected strains included an adjustment of the transcriptome to repress genes involved in ribosome biogenesis and an induction of genes participating in energy production and programmed cell death (Park et al., 2020, Applen Clancey et al., 2020). The authors then went one step further and investigated the viral influence on fungal pathogenicity by treating bone marrow-derived dendritic cells (BMDC) with virus-infected and cured *M. restricta* strains or protein components of the viral particles. Surprisingly, the BMDCs displayed an induction of TLR3 expression as soon as they were

treated with the infected fungi. Moreover, the cytokines tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), IL-10, interferon alpha (IFN-α), and IFN-γ were elevated in a TLR3-dependent manner, underlining the major role of the mycoviral dsRNA as a PAMP recognized by the innate immune system during *M. restricta* infection (Park et al., 2020). A separate study focusing on a related species, *M. sympodialis*, again infected with a dsRNA virus of the *Totiviridae* family termed MsMV1, reported a different outcome regarding fungal pathogenicity. Here, the authors used bone marrow-derived macrophages (BMDM) to test whether virus-infected fungus triggered activation via TLR3. The BMDMs displayed an induction of IFN-β upon infection with the virus-infected fungus in contrast to the virus-cured fungus. But the activation of the macrophages appeared to happen in a TLR3-independent manner, raising the question of what receptor might be mediating the IFN-β induction (Applen Clancey et al., 2020). The authors suggested that other dsRNA-dependent PRRs (e.g., RIG-I or MDA5, see following section) might contribute to interferon expression, although the effect of viral proteins like RNA-dependent RNA polymerases or capsid proteins on macrophage activation were not tested as in the Park study (Park et al., 2020).

The examples discussed intriguingly show how TLR3 can contribute to innate immune activation by the detection of either fungal or mycoviral RNAs, albeit the relevance of TLR3 changes according to the fungal pathogen. Similarly, *P. brasiliensis*, the cause of paracoccidioidomycosis, escapes the immune response by negative regulation of the activity of TLR3 during infection (Jannuzzi et al., 2019). Deletion of TLR3 in murine BMDMs resulted in increased fungicidal activity, reduced fungal burden, and elevated production of nitric oxide. Moreover, intratracheal infection of wild-type or TLR3-deficient mice with *P. brasiliensis* caused an increase in $CD8^+$ T cells in the knockout, which an earlier study showed to be important for resolution of murine paracoccidioidomycosis (Jannuzzi et al., 2015). The increase of these T cells concomitantly occurred with an induction of the cytokines IL-17, IL-1β, IFN-γ, and IL-6, all of which are known to contribute to defense against fungal infections (Jannuzzi et al., 2015, Alegre-Maller et al., 2014, de Souza Silva et al., 2015, Morais et al., 2016). Although this study did not investigate the molecules responsible for the pathogen escape mechanism, it nicely illustrates how PRRs can be exploited during infections to prevent clearance of fungi by the host immune system.

A similar effect was observed in a murine macrophage-infection model investigating the contribution of the ssRNA receptor TLR7 to defense against invasive pulmonary *Aspergillosis* (IPA) caused by *A. fumigatus* (Xu et al., 2021). In contrast to the upregulation of *TLR7* expression during IPA, deletion of the receptor resulted in a reduction of the fungal burden, lung damage, and inflammatory response as well as a decreased mortality of the knockout compared to the wild-type mice, indicating a negative regulatory role of the receptor during *A. fumigatus* infection. Specific activation of TLR7 by the compound R837 increased the mortality of mice infected with *A. fumigatus* in an alveolar macrophage-dependent manner. According to the data, TLR7 activity negatively regulated phagocytosis and the generation of reactive oxygen species leading to a higher susceptibility to invasive pulmonary aspergillosis (Xu et al., 2021).

The outcome of TLR7 activity is highly dependent on the invading fungal pathogen as well as the immune cell responding to those pathogens. An interesting example for this observation was given in a report deciphering the TLRs involved in the innate immune response to *C. albicans* infection (Biondo et al., 2012). As mentioned in the beginning of this chapter, TLR2 and TLR4 play an integral role in the recognition of fungal cell wall components of *C. albicans* (Bellocchio et al., 2004, Mambula et al., 2002, Meier et al., 2003, Netea et al., 2002). Biondo and colleagues aimed to expand these findings by investigating the role of nucleic acid dependent PRRs by focusing on the role of TLR7 (and TLR9). For this, they utilized mouse BMDMs and BMDCs, which were not only challenged with

purified yeast RNA but also with whole yeast cells to reflect a more natural infection situation. The treatment with purified *C. albicans* RNA activated BMDCs but not BMDMs and led to an induction of the cytokines IL-12p70, IL-23, and TNFα in a TLR7-dependent manner. Moreover, downstream effectors like the TLR-hub protein MyD88 (Takeda et al., 2003, Saikh, 2021) and transcription factor IRF1 (Feng et al., 2021) were clearly important for the induction of IL-12p70, while IL-23 and TNFα expression seemed to rely on a different transcription factor and even on different receptors when the BMDCs were challenged with whole cells. *TLR7*-deficient BMDCs infected with live *C. albicans* cells only resulted in a depletion of IL-12p70, while IL-23 and TNFα generation were still increased, indicating different receptor dependencies for the cytokine induction and a more concerted inflammatory response of the different PRRs. The appropriate innate immune activation depended on the correct localization of the endosome-situated TLRs since interference with this process by mutating UNC93B1, important for recruitment of TLR3, TLR7/8, and TLR9 to the endosome, ablated cytokine induction, demonstrating that the ssRNA ligand had to be released from internalized/phagocytosed pathogens for proper recognition (Tabeta et al., 2006, Brinkmann et al., 2007, Kim et al., 2008). These findings were recapitulated in mice lacking TLR7, MyD88, IRF1, or functional UNC93B1 strengthening the importance of this inflammatory response axis in candidiasis (Biondo et al., 2012).

Similar results were later obtained regarding the importance of the TLR7/MyD88 axis in the innate immune response upon infection with *H. capsulatum* in a murine infection model utilizing BMDMs and BMDCs (Van Prooyen et al., 2016). Conidia of this dimorphic fungal pathogen enter the human lung and then disseminate into other tissues and organs (Woods, 2003, Nosanchuk and Gacser, 2008). Controlling *H. capsulatum* infection requires induction of T cells to produce TNFα and IFN-γ, which activate phagocytic cells like macrophages. Macrophages are responsible for phagocytosis and clearance of the pathogen, but are also exploited by the fungus for replication in the phagolysosome (Deepe, 1988, Nosanchuk and Gacser, 2008). In contrast, DCs are capable of phagocytosing and killing *H. capsulatum* to support an adequate immune response through stimulation of $CD8^+$ T cells (Nosanchuk and Gacser, 2008, Gildea et al., 2001, Lin et al., 2005). In line with this notion, the investigation of Van Prooyen et al. focused on the immune responses orchestrated by BMDCs compared to BMDMs. Interestingly, *H. capsulatum* infection of the dendritic cells, but not the macrophages, resulted in an induction of type I IFNs that was dependent on TLR7/TLR9 and MyD88. Combined deletion of both receptors completely ablated IFN-I production *in vitro* in BMDCs and *in vivo* in mice, leading to a higher neutrophil recruitment, lung damage, and mortality rate. The lack of TLR7/TLR9-dependent IFN-I production affected the $CD4^+$ and $CD8^+$ T cell-controlled IFN-γ expression, a known factor in *H. capsulatum* infection control (Wu-Hsieh and Howard, 1987, Allendoerfer and Deepe, 1997). These studies clearly show that RNA recognizing-PRRs play an intricate role in both defense against fungal infection and as a point of exploitation by fungal pathogens against host organisms. Additional work will be required to determine how other organisms have evolved to control or even exploit these pathways, and how these signaling cascades might be utilized for antifungal drug development.

B. RIG-I-like receptors and the antifungal response

Other RNA-dependent receptors like the retinoic acid-inducible gene I (RIG-I)-like receptors (RLR) are produced and localized to the cytoplasm in many cell types. This class comprises of three proteins, RIG-I, melanoma differentiation-associated protein 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2), which are all characterized by a central helicase domain and a carboxy-terminal domain (Rehwinkel and Gack, 2020). RIG-I and MDA5 also contain two N-terminal caspase activation

and recruitment domains (CARDs) that upon RNA-oligomerization and activation are necessary for the interaction with the CARD of mitochondrial antiviral-signaling protein (MAVS) (Kawai et al., 2005, Meylan et al., 2005, Seth et al., 2005, Xu et al., 2005, McWhirter et al., 2005, Hou et al., 2011). MAVS in turn mediates activation of TANK-binding kinase 1 (TBK1), IκB kinase-ε (IKKε), IKKα, and IKKβ, allowing for activation of IFN regulatory factor 3 (IRF3), IRF7, and nuclear kappa-light-chain enhancer of activated B cells (NF-κB). These three transcription factors promote expression of antiviral genes upon translocation to the nucleus (Goubau et al., 2013, Hartmann, 2017, Rehwinkel and Gack, 2020). The RLR pathway is induced upon interaction of RIG-I and/or MDA5 with RNA molecules possessing specific features that are normally not found on endogenous RNAs. RIG-I recognizes uncapped 5´-di- or triphosphates of blunt-end, dsRNA segments lacking 2´-O-methylations at the 5´-ends (Rehwinkel and Gack, 2020). Soon after its description, the RLR pathway was found to be essential in the defense against viral infections (Yoneyama et al., 2004, Gitlin et al., 2006, Kato et al., 2006) and in innate immunity against bacterial infections (Abdullah et al., 2012, Zou et al., 2016, Sahr et al., 2022). The RNA features activating MDA5 remain somewhat ambiguous, but in general very long (>2000 base pair) dsRNA-molecules seem capable of activating the receptor (Takeuchi and Akira, 2010, Goubau et al., 2013, Rehwinkel and Gack, 2020).

Reports have also revealed a role for these same PRRs in the detection of fungal pathogens. A study from 2015 investigated changes of the transcriptome of macrophages and peripheral blood mononuclear cells to evaluate gene expression in response to *C. albicans* infections (Jaeger et al., 2015). Wild-type or *Δhgc1* (yeast-locked) *C. albicans* cells were used to infect macrophages for 4 or 24 hours followed by microarray analysis to assess gene expression during infection. 62 macrophage genes were strongly induced after challenge with wild-type hyphae-forming fungus, predominantly from the IFN signaling pathway, but four genes could be assigned to the RLR signaling cascade, namely *IFIH1* (*MDA5*), *ISG15*, *IL-8*, and *TRIM25* (Jaeger et al., 2015, Loo and Gale, 2011, Rehwinkel and Gack, 2020). As a major receptor of RLR signaling, MDA5 was further investigated to determine its role during *Candida* infection. Expression analysis of peripheral blood mononuclear cells stimulated with either heat-killed yeast or hyphae compared to LPS-treatment or *Borrelia burgdorferi* infection as controls confirmed a strong induction of *MDA5* in the immune cells (Jaeger et al., 2015), and another study showed that MDA5 enhances invasive *C. albicans* infection by regulating apoptosis and phagocytosis in macrophages (Chen et al., 2023). The importance of *MDA5* was further evaluated in human patients suffering from candidemia by investigation of SNPs occurring mostly in genomic regions involved in immune-mediated diseases (Jaeger et al., 2015). Among other *MDA5*-SNPs associated with increased susceptibility to *Candida* infections, missense mutations of Ala946Thr and His843Arg (rs1990760 and rs3747517) resulted in the clearest dysfunctionalities of RLR signaling, manifesting as dysregulation of cytokine signaling in an *in vitro* model. The authors proposed that these missense SNPs might corrupt the interaction of MDA5 with dsRNA (Ala946Thr and His843Arg) and interfere with the interaction of receptor monomers (His843Arg), which are known to form filaments along the recognized RNA necessary for downstream MAVS interaction (Jaeger et al., 2015, van der Lee et al., 2014, Hou et al., 2011, Wu et al., 2013). Recapitulation of *Candida* yeast or hyphae infection with murine splenocytes with or without *MDA5*/*IFIH1* deletion only hinted to a similar role of the receptor in mice, suggesting other pathways may be required in other organisms. Nevertheless, this data conclusively indicated an important role for MDA5 in the defense against candidiasis. In the future, it will be interesting to see how the presence of fungal dsRNA is influenced by the activity of other host processes, such as modification by host dsRNA-modifying enzymes like the adenosine deaminase that acts on RNA (ADAR) proteins, which were previously shown to be activated during *C. albicans* infection (Huang et al., 2018).

Further investigations of the RLR pathway in response to *A. fumigatus* infections expanded on the importance of MDA5 in anti-fungal immunity. Deletion of *MDA5* or *MAVS* resulted in a higher susceptibility of mice upon challenge with *A. fumigatus*, which was represented by decreased conidial killing by neutrophils, altered cytokine production, increased lung damage, and ultimately a higher mortality rate. Infection of primary murine fibroblasts supported this by showing that viable conidia or even isolated dsRNA of *A. fumigatus* could lead to the induction of type I and III IFNs, which depended on the action of MDA5 and MAVS (Wang et al., 2020). The importance of both RLR-pathway effectors became even more visible in a follow-up study assessing the risk factors of hematopoietic stem-cell transplantation recipients affected by IPA, where SNPs in MDA5 and MAVS associated with a higher risk of IPA. A clear connection was shown between the missense SNP rs1990760 (Ala946Thr) in *MDA5* and susceptibility to *A. fumigatus* infection, indicating a similar effect of the same mutation not only for IPA but also candidiasis (Wang et al., 2022, Jaeger et al., 2015). Moreover, the SNP rs17857295 in *MAVS* appeared to contribute to a higher IPA-development risk as well. While the identified *MDA5* mutation and increased IPA-risk was associated to the recipient, this was reversed for the *MAVS* SNP displaying a link to the donor. Underlining the importance of MAVS functionality in a hematopoietic stem-cell transplantation context, *A. fumigatus* infection was recapitulated in mice devoid of *MAVS* in $CD11c^{+}$ cells, revealing alveolar macrophages as largely responsible for antifungal host resistance (Wang et al., 2022). These findings indicate a critical role of the RLR pathway in immunity against some of the most prevalent fungal pathogens, which will likely be expanded to other microbes in future investigations.

C. Transcriptomic and non-coding RNA responses to fungal infections

The last sections gave an overview of the role of RNA-dependent PRRs in the regulation of the antifungal immune response, where specific fungal RNA was shown to activate TLR or RLR pathways and induce appropriate innate defense mechanisms. In addition to these immunological studies, several recent reports have investigated how fungi control the coding and non-coding RNA landscape of hosts by affecting expression, functionality, and even host immune regulation. One approach to assess the transcriptional changes induced during fungal infection in diverse immune cells is to perform RNA-seq of the host and pathogen transcriptomes simultaneously, as was reported recently using cytomegalovirus (CMV) together with *A. fumigatus* to infect monocyte-derived dendritic cells (moDCs)(Seelbinder et al., 2020). The authors compared the transcriptomes obtained from untreated moDCs or *A. fumigatus* to infections of the host cells with either the fungus or CMV in co-infection experiments. The transcriptional changes documented in the singular infections of moDCs reflected the previously described transcriptome adjustments induced upon *A. fumigatus* or CMV stimulation. Fungal infection promoted TNF signaling, which induced NFκB-dependent gene expression in addition to *IL-1B* and *IL-10*, whereas CMV infection strongly elevated signaling of RIG-I, cGAS, and STING pathways as well as *TLR3*, *CXCL10, IFN-B,* and *ZBP1* expression (Seelbinder et al., 2020, Yang et al., 2020, Onomoto et al., 2010). The commonality between all of these signaling responses was that they were strongly downregulated when the moDCs were co-infected with both pathogens, indicating a synergistic effect on host immunity that benefits both the fungus and the virus to the detriment of the host.

Another compelling example of dual RNA-seq usage came from the study of *C. albicans* during infection of BMDMs (Munoz et al., 2019), where the authors aimed to resolve the transcriptional changes occurring during host-pathogen interaction and apply single-cell RNA sequencing to distinguish different subpopulations with unique single-cell transcriptomes. In doing so, they found significant regulatory changes in the transcriptome over time for both the macrophages and the

fungus. While the macrophages heavily induced proinflammatory cytokine production and expression of several transmembrane receptors (for fungal recognition) at early timepoints after infection, the internalized live fungal pathogens rapidly adjusted metabolic genes and pathways likely to adapt to the scarce nutrient availability in the phagolysosomal environment. After four hours of infection, repressed fungal gene clusters became more transcriptionally active, including those involved in translation, cell wall remodeling, glucose, and nucleoside metabolism. Interestingly, BMDMs started to repress immune response-relevant genes at later timepoints, including phagocytosis signaling, proinflammatory cytokines, and transmembrane receptors as part of a transcriptional shift towards a reduced immune response. Using single-cell resolution for RNA-seq, the authors correlated late transcriptional changes of the macrophages to the induction of metabolic and filamentation pathways in *C. albicans*, suggesting that the pathogen might drive the transcriptional shift in the host cells. Hence, these data indicated a coordinated transcriptional adjustment during the host-pathogen interaction by both organisms.

Together with the application of RNA-seq to investigate expressional changes of protein-coding RNAs, efforts have also been invested in defining the response of the non-coding transcriptome. The focus of these studies has been the miRNAs due to their defined regulatory role in gene expression via RNAi. In order to investigate possible functionality of miRNAs during infection, a murine alveolar macrophage cell line model system challenged with *A. fumigatus* was utilized to investigate the influence of a particular miRNA on the expression of TLR2 and how this connects to the induction of autophagy (Wu et al., 2019). As one of the major PRRs responsible to detect *A. fumigatus*, TLR2 contributes to the activation and coordination of the immune response (Bellocchio et al., 2004, Chai et al., 2009, Mambula et al., 2002). Further work established that the regulation of this receptor depended on miRNA-344b-1-3p modulation of TLR2 expression (Xu et al., 2017). This regulatory relationship displayed an inversely proportional expression pattern resulting in the decrease of miRNA levels while the *TLR2*-mRNA abundance increased during *A. fumigatus* infection (Wu et al., 2019). Accordingly, miRNA-dependent regulation of *TLR2* correlated with downstream pathways, including the autophagy response necessary to cope with the fungal pathogen. Similarly, the importance of miRNAs for the anti-fungal immune response was also shown in a broader experimental set-up investigating small RNA expression patterns in human DCs responding to *A. fumigatus* or *C. albicans* challenge (Dix et al., 2017). By employing small RNA-seq, the authors identified three (after 6 h; miR-132-3p, miR-132-5p, miR-212-5p) and five (after 12 h; miR-132-3p, miR-132-5p, miR-129-5p, miR-212-3p, miR-212-5p) differentially expressed miRNAs that were predicted to target various immune response genes (e.g., *FKBP1B*, *BTN3A2K*). Successful verification of several predicted targets by knockdown of the respective miRNAs underlined the value of the high-throughput findings. Thus, this data supported a scenario in which small RNA expression is adjusted during host-fungal interaction, indicating a more pronounced function as a regulatory element and complementing the growing body of literature reporting similar effects for other cell types (see (Boomiraj et al., 2015, Croston et al., 2018)) and non-coding transcripts like lncRNAs (e.g. (Riege et al., 2017)).

D. Interkingdom communication

As has been mentioned in numerous examples above, the interplay between fungi and animal hosts is highly regulated and complex. In this section we have focused primarily on the sensor proteins that detect an invading organism and the regulation of RNA in response to activation of these sensors but have barely scratched the surface of the RNA molecules themselves that are transferred between fungi and animals. Although only an emerging field, several examples already exist of RNAs that are trafficked between organisms during infection. These come primarily from studies of plant-fungal pathogens, but other examples do exist in animals. In insects, miRNAs of the let-7 family and others

like miR-100 were shown to be transferred to the entomopathogenic fungus *B. bassiana* from the silkworm *Bombyx mori* and the mosquito *A. stephensi* to inhibit expression of the fungal virulence-related genes *sec2p* and *C6TF* (**Fig. 3**) (Wang et al., 2021). The induction of let-7 and miR-100 by the host organism is initiated upon penetration of the hemocoel by the fungus, resulting in decreased production of fungal Sec2p and C6TF and ultimately decreased virulence. Exploiting these findings, the authors then showed that expression of an anti-miR-100 miRNA sponge in the fungus led to increased virulence of the pathogen. The clear implication of this experiment is that a more pathogenic form of *B. bassiana* may hold potential as a biocontrol agent against insect pests in some cases. Of course, additional careful work needs to be done to ensure that such strategies do not have unforeseen consequences.

In humans, *C. albicans* induces miRNA trafficking by host monocytes to influence fungal behavior and elicit a growth advantage (Halder et al., 2022, Halder et al., 2020). In this case, the miRNAs seem to be associated with extracellular vesicles, although the exact mechanism of loading into vesicles and delivery into the fungus remains unclear. Experiments using naked miRNAs also resulted in a growth advantage to the fungus, suggesting that antifungal RNA delivery to *C. albicans* could be a strategy for treatment in the future. Other systems have also investigated the transfer of host cargo molecules by extracellular vesicles, as with human neutrophils and the filamentous pathogen *A. fumigatus* (Shopova et al., 2020, Rafiq et al., 2022). In this case, neutrophils have an inhibitory effect on fungal growth, like that observed in the example from mosquitos above. More work will have to be done to define the RNA cargo trafficked in these vesicles, and how these RNAs might influence the outcome of fungal infection in different patient populations.

Other examples of cross-kingdom RNA interference, or even novel regulatory mechanisms, will most certainly be observed between fungi and their hosts. The large-scale transcriptional responses to most pathogens may hide some of these regulatory features amongst so many changes, but we do know of cases where regulation seems to be manipulated by the fungal pathogen, as with macrophage polarization after *C. neoformans* infection (Subramani et al., 2020). Through an as-yet-unknown mechanism, *C. neoformans* appears to direct macrophages towards an immature, unpolarized state, although more work will be needed to confirm that these regulatory mechanisms also occur *in vivo* during infection. By combining high-quality dual transcriptomic datasets (Niemiec et al., 2017, Munoz et al., 2019) with more rigorous biochemical and genetic characterizations, we are likely to uncover all sorts of new regulatory features previously hidden from view.

Section V. Conclusions: Exploiting RNA Biology for Therapy

Our increased collective knowledge of RNA biochemistry and regulation is now being translated to more applied projects, with the hope of leveraging what we have learned for the treatment of fungal pathogens with novel therapeutics. Although a field in its infancy, the examples highlighted throughout this chapter provide numerous target points for development of creative, new therapeutics. Arguably one of the first examples of leveraging these basic molecular biological principles for therapeutics came from the nucleoside analog, 5-fluorocytosine, which is converted into 5-fluorouridine in susceptible fungi to inhibit protein and DNA synthesis (Vermes et al., 2000). Unfortunately, high rates of resistance currently limit the overall effectiveness of this class of antifungals (Billmyre et al., 2020), but it remains in use in specific cases.

More recent approaches have tried to leverage chemicals designed to inhibit important RNA molecules, particularly group II intron ribozyme structures found in essential genes of fungi (Liu and Pyle, 2021). A small molecule inhibitor was also recently demonstrated to exhibit target specificity and catalytic activity inhibition of the ribozyme against the human pathogen *C. parapsilosis*

(Fedorova et al., 2018). Another approach is to design RNA molecules that fit into a particular protein to elicit an effect, so called RNA aptamers. Although limited examples of RNA aptamers exist in general, they hold therapeutic promise and have already been designed to facilitate disassembly of *C. albicans* biofilms (Bachtiar et al., 2019). Antisense oligonucleotides have been explored for control of *C. albicans* filamentation with some success (Araujo et al., 2019). For this a 2'-OMethylRNA antisense oligonucleotide (ASO) was used to target the *EFG1* mRNA to limit gene expression and translation of Efgp1, resulting in decreased filamentation and virulence. Previously unexplored therapeutic targets are also being investigated, as is the case with the Tpt1 enzymes found in fungi and plants that are absent from humans (Dantuluri et al., 2021). These essential enzymes are important for tRNA splicing and offer a potential target for novel antifungal therapy building on our improved understanding of basic molecular biological mechanisms in human fungal pathogens. Other approaches could utilize this knowledge by employing other classes of small RNAs beside ASOs to modulate the gene expression patterns of fungal pathogens and influence the outcome of infection. For instance, using small RNA molecules like siRNAs or miRNAs to specifically target certain virulence relevant genes by hijacking the fungal RNAi machinery could be an elegant way to suppress fungal infections. A proof of concept for this strategy was reported using *A. fumigatus* strains expressing inverted-repeat transgenes as a source for siRNAs targeting mRNAs of either *pksP* or the conditionally essential gene *pabA* (Kelani et al., 2023). Both strategies resulted in efficient knock-down of the respective gene and growth reduction, although decrease of the latter mRNA resulted in a more severe decrease. Thus, targeted RNAi dependent approaches could be an interesting treatment option for specific fungal infections although efficient uptake of siRNAs still faces challenges (see below) (Bruch et al., 2022). In this regard, the agricultural sector might serve as a role model due to recent advances in exploiting the RNAi system and cross-kingdom communication between plant-pathogens and the host (Cai et al., 2018a). Strategies involving the use of transgenic plants expressing anti-pathogen small RNAs (host-induced gene silencing) or spray-treatment with RNA of the plant host (spray-induced gene silencing) proved to be efficient in infection prevention (Cai et al., 2018a, Cai et al., 2019). Translation of these options into antifungal medication could advance our repertoire tremendously for treatment of patients suffering from severe fungal infections (Cai et al., 2019).

These approaches offer unique advantages for treatment of fungal infections over more conventional approaches. The target space over chemical inhibitors is much wider, meaning that RNA drugs can be designed against targets more specifically, even in highly conserved pathways. In addition, RNA is easy to synthesize; fast to adapt to evolving pathogens, and easy to redesign upon development of antifungal resistance. Many difficulties will have to be overcome to employ such drugs against human fungal pathogens, but innovative strategies are already in development. By leveraging the biochemical and biological solutions being employed in related systems, we are poised for rapid advances of these therapeutics against a wide range of pathogens. Advances like those embraced by the mRNA vaccines against SARS-CoV-2 are the most obvious, such as replacement of uridine with modified N1-methyl-pseudouridine to limit recognition and activation of host nucleic acid sensors like those discussed above (Corbett et al., 2020, Kariko et al., 2012, Anderson et al., 2010). Improvements in RNA synthesis and *in vitro* transcription have decreased unwanted immunogenic byproducts like dsRNA (Nelson et al., 2020), further facilitating this new generation of therapeutics.

The largest barrier to exploitation of RNA regulatory pathways in fungi by RNA therapeutics is likely the formidable fungal cell wall (Bruch et al., 2022). Here, leveraging approaches developed against other human diseases may not be entirely possible, as the fungal cell wall has unique

properties compared to a human cell. There is hope that protection of RNA in modified liposomes and nanoparticles or conjugation to aptamers and antibodies may result in improved delivery to cells (Dammes and Peer, 2020, Howard et al., 2017, Moustafa et al., 2021), but the utility of these strategies in traversing the fungal cell wall remains unstudied. Approaches using engineered extracellular vesicles may hold promise (Zhang et al., 2020), but are far from applicable in the clinic currently. Recent studies showing that host-derived extracellular vesicles respond and influence the course of infection with *A. fumigatus*, *C. albicans,* and *C. neoformans* suggest that modulation of human fungal pathogens is possible (Shopova et al., 2020, Halder et al., 2020, Zhang et al., 2021). Finally, in the absence of direct antifungal strategies, it is possible that more indirect approaches reliant on improving the remaining host immune response may prove valuable (de Candia et al., 2016). Only the future will tell how we can apply our increasing knowledge of RNA in host-fungal pathogenesis for the betterment of human health.

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VIII. Index

Figure Legends

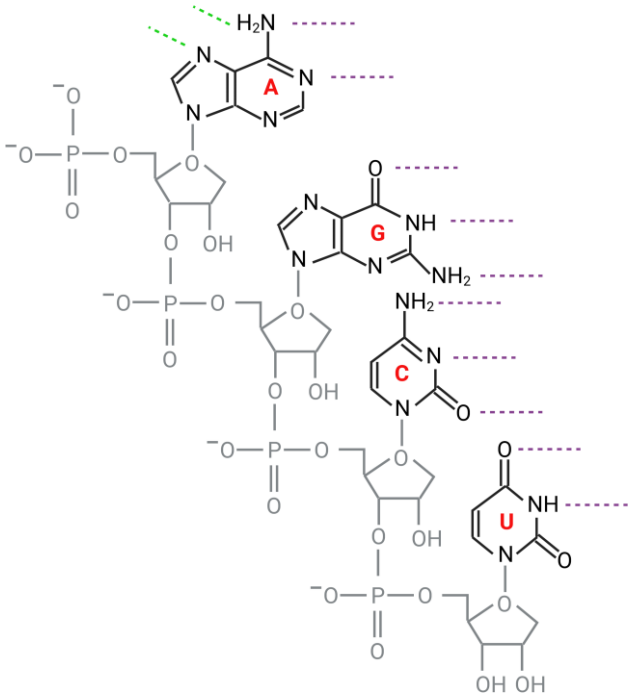

Fig. 1. Chemical structure of RNA showing the sugar phosphate backbone in gray and the nitrogenous bases adenine (A), guanine (G), cytosine (C), and uracil (U) in black. The Watson-Crick base-pairing interactions are highlighted in purple with an example of Hoogsteen pairing shown in green. Created with BioRender.com.

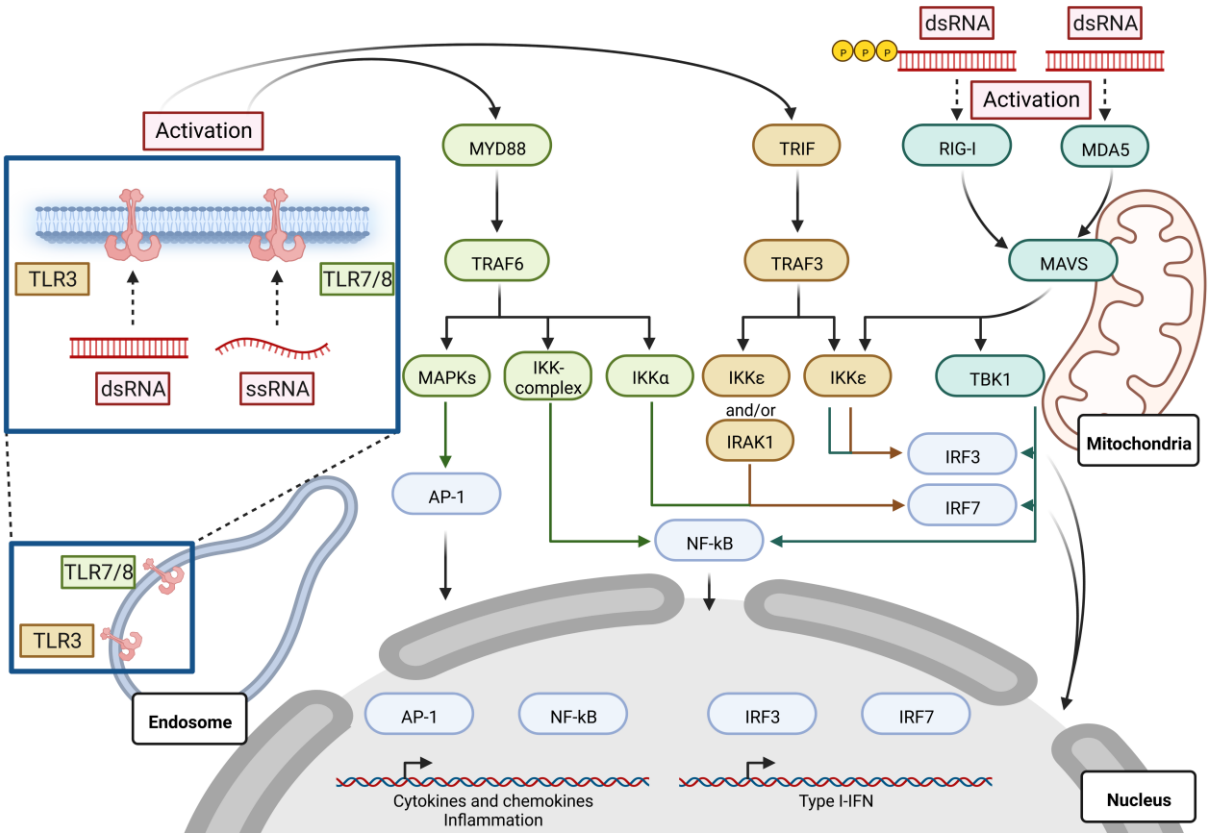

Fig. 2. Innate immune signaling cascades of RNA-dependent PRRs activated by fungal pathogens. Sensing of various foreign (fungal) RNAs like dsRNAs or ssRNA is achieved by the endosomal receptors TLR3 or TLR7/8 and cytoplasm-localized RIG-I or MDA5. Signaling is mediated by the TLR3/TRIF axis (orange boxes), TLR7/8/MYD88 axis (green boxes), and/or RIG I/MDA5/MAVS axis (dark green boxes) by employing the transcription factors AP-1, NFκB, IRF3 and, IRF7 for the activation of diverse innate immunity genes. Adapted from (Rehwinkel and Gack, 2020, Jannuzzi et al., 2020, Uehata and Takeuchi, 2020). Created with BioRender.com.

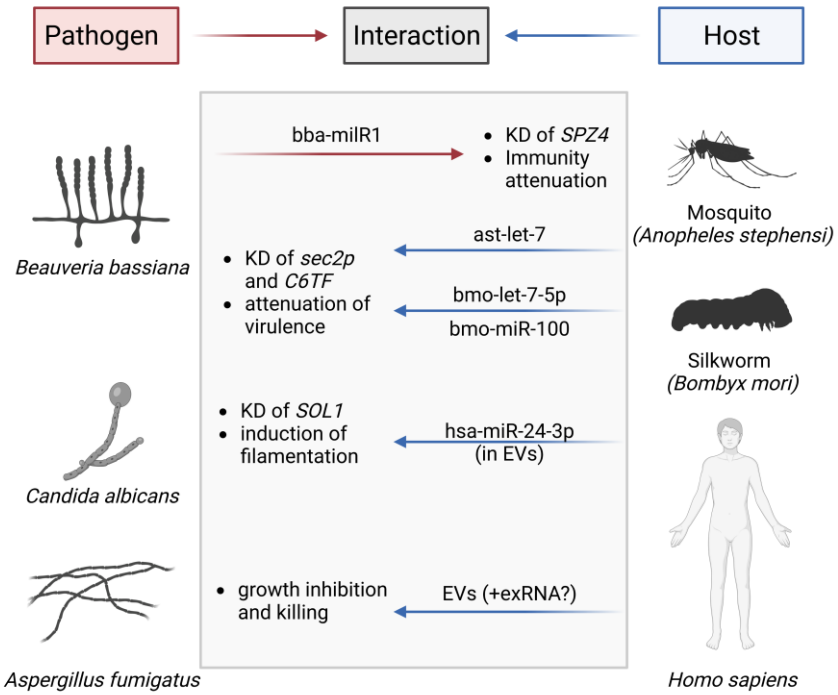

Fig. 3. Cross-kingdom RNA trafficking modulates virulence and immunity. The depicted examples show reported cross-kingdom interactions (grey box) of fungal pathogens and their respective hosts. If identified, trafficked small RNAs are given together with the described outcome in the target species (red or blue arrows). KD, knockdown; EVs, extracellular vesicles. Created with BioRender.com.