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RNAi pathways in *Mucor*: A tale of proteins, small RNAs and functional diversity

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ABSTRACT

The existence of an RNA-mediated silencing mechanism in the opportunistic fungal pathogen *Mucor circinelloides* was first described in the early 2000. Since then, *Mucor* has reached an outstanding position within the fungal kingdom as a model system to achieve a deeper understanding of regulation of endogenous functions by the RNA interference (RNAi) machinery. *M. circinelloides* combines diverse components of its RNAi machinery to carry out functions not only limited to the defense against invasive nucleic acids, but also to regulate expression of its own genes by producing different classes of endogenous small RNA molecules (esRNAs). The recent discovery of a novel RNase that participates in a new RNA degradation pathway adds more elements to the gene silencing-mediated regulation. This review focuses on esRNAs in *M. circinelloides*, the different pathways involved in their biogenesis, and their roles in regulating specific physiological and developmental processes in response to environmental signals, highlighting the complexity of silencing-mediated regulation in fungi.

1. Introduction

The discovery of RNA-mediated gene silencing in the late twentieth century caused a major impact in the gene expression regulation world. Conceptually described first in plants and fungi (Napoli et al., 1990; Romano and Macino, 1992), early experiments that clarified the triggering mechanism in nematodes (Fire et al., 1998) gave way to a myriad of works describing the presence of this silencing mechanism in most eukaryotic organisms (Cerutti and Casas-Mollano, 2006). The overall landscape that now draws all this information on gene silencing mechanisms is really amazing. Gene silencing was initially described as a mechanism of defense against invasive nucleic acids and viruses, with a few components participating in the machinery that generates the small interfering RNA (siRNA) molecules which are the signature of the gene silencing mechanism. In most eukaryotic systems the basic silencing machinery consists in an RNase III protein (Dicer) that processes dsRNA precursors into siRNAs, and an Argonaute protein that uses siRNAs to guide selective destruction of target mRNAs. Additionally, some silencing-competent organisms, including plants, nematodes and fungi, require an RNA-dependent RNA polymerase (RdRP) protein to generate dsRNA from single-stranded RNA inducers or to amplify siRNA signals (Ghildiyal and Zamore, 2009; Garre et al., 2014). Besides these siRNAs, multiple classes of endogenous small RNA (esRNA) molecules, including microRNAs (miRNAs) that act as riboregulators controlling a wide panoply of cellular processes, have been identified in both metazoans and lower eukaryotic organisms (Fabian et al., 2010). Biogenesis of most of those esRNAs shares a minimal common silencing machinery, named RNAi machinery, which comprises Dicer and Argonaute proteins. In addition to this basic or canonical pathway, different non-canonical alternatives in which Dicer proteins do not participate have been described. These non-canonical pathways are responsible for the biogenesis of specific esRNAs, not only the well-known Piwi-interacting RNAs (piRNAs) but also miRNAs and miRNA-like (milRNA) molecules (Senti and Brennecke, 2010; Chang et al., 2012). In these cases, the catalytic activity of Argonaute family proteins and the trimming activity of specific exonucleases are required to produce mature esRNAs. Nevertheless, most of non-canonical miRNA molecules are poorly conserved and low in abundance, which sheds doubt on their functionality.

Among filamentous fungi, putative regulatory esRNAs produced by canonical and non-canonical pathways were first described in *Neurospora crassa* and *Mucor circinelloides* (Lee et al., 2010a; Nicolás et al., 2010). Several lines of evidences suggest that *Neurospora* milRNAs could regulate gene expression in a similar way to animal and plant miRNAs, although it seems that they do not play critical roles in vegetative growth or developmental processes, since *Neurospora* milRNA knock-out strains do not show any vege-

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tative defect (Chang et al., 2012). However, the sterile phenotype in homozygous crosses shown by *Neurospora* mutants of RNAi genes required for meiotic silencing by unpaired DNA (MSUD), a RNAi-related pathway that operates during meiosis, may suggest a role of milRNAs in the regulation of sexual development (Alexander et al., 2008; Xiao et al., 2010; Hammond et al., 2011). The presence of milRNAs has been predicted in other fungi, although further functional research is necessary to evaluate their biological roles (Goodwin et al., 2011; Jiang et al., 2012; Zhou et al., 2012; Kang K et al., 2013; Lau et al., 2013; Lin et al., 2015; Dahlmann and Kück, 2015).

M. circinelloides, a basal fungus belonging to the subphylum Mucoromycotina, has achieved a major place as a fungal model system for studying different molecular processes. This has been mainly due to its distinctive position in the fungal tree of life, since this basal lineage of the fungal kingdom is evolutionary distant from other fungal models widely used for molecular studies, such as *N. crassa*, *Aspergillus nidulans* or *Cryptococcus neoformans*, which belong to the subkingdom Dikarya. Furthermore, *Mucor* is the basal fungus where more molecular tools are available, including functional analysis by RNAi. The value of *M. circinelloides* as a model has been further enhanced recently, since it has emerged as an opportunistic human pathogen, a causal agent of the rare but lethal infection mucormycosis. This emerging infection, which is caused by several fungi of the order Mucorales (Chayakulkeeree et al., 2006), mostly affects immunocompromised patients, and it presents a very high mortality rate, going up to >90% in disseminated infections (Spellberg et al., 2005). In the last years, an increasing number of cases of mucormycosis have been described in immunocompetent/otherwise healthy individuals, which has raised the alarm on this emerging disease (Fanfair et al., 2012; Lee et al., 2014).

M. circinelloides, which has been used as a model for studying gene silencing (Garre et al., 2014), combines basic elements of its RNAi machinery to produce different classes of esRNAs that regulate the expression of their own genes (Nicolás et al., 2010; Cervantes et al., 2013). The recent discovery of a new silencing pathway involved in the specific degradation of endogenous mRNAs, and the participation of a novel RNase III protein in this pathway (Trieu et al., 2015), adds more elements to this wide and fascinating world of gene silencing-mediated regulation. In this review we summarize our current knowledge on the pathways involved in the biogenesis of esRNA molecules and the functions which are presumably regulated by these esRNAs, providing a general overview on the regulatory role of the gene silencing machineries in significant biological processes of *Mucor*, including pathogenesis.

2. Gene silencing in *Mucor*

M. circinelloides is the only basal fungus in which gene silencing has been molecularly and functionally characterized, although the presence of all the key proteins of the silencing machinery in other Mucorales suggests that this mechanism may be operative in other basal fungi (Garre et al., 2014). Although the first reference on the existence of a gene silencing mechanism in *Mucor* dates back more than a decade (Nicolás et al., 2003), it has not been until more recently when the variety of esRNAs and the complexity of the silencing pathways involved in their biogenesis have become evident. To reach this knowledge, identification and functional characterization of the main genes involved in the gene silencing pathway induced by exogenous transgenes have been essential. Briefly, RNA-dependent RNA polymerase RdRP-1 participates in the initiation step of the silencing mechanism, since it is essential for producing antisense RNA from transgene transcripts (Calo et al., 2012). The role of RdRP-1 in gene silencing is similar to that of *N. crassa* QDE-1 protein, which is required for induction of silencing by sense transgenes (Cogoni and Macino, 1999; Goldoni et al., 2004) by generating aberrant RNA from DNA strands and converting it into dsRNA (Lee et al., 2010b). This dual polymerase activity seems to be unusual, since QDE-1 and RdRP-1 belong to the γ family of RdRP proteins, whereas most of RdRPs from the three eukaryotic kingdoms belong to class α (Zong et al., 2009). Triggering dsRNA molecules produced by RdRP-1 are then processed by the main RNase III endonuclease Dicer-like (Dcl) protein, Dcl-2, into two different classes of siRNAs, 21 and 25 nt long (de Haro et al., 2009). There is another Dicer enzyme, Dcl-1, which does not have a relevant role in transgene-induced silencing but is partially redundant with Dcl-2 (Nicolás et al., 2007; de Haro et al., 2009). Only one out of three Argonaute (Ago) endonucleases found in *M. circinelloides*, Ago1, is required for silencing, at least during vegetative growth, by binding siRNA molecules and provoking degradation of target transcripts (Cervantes et al., 2013). Finally, a different RdRP protein, RdRP-2, is essential for amplification of the silencing signal, a process identified in some fungi, plants and nematodes that produces new dsRNA molecules by using processed target RNAs as template (Calo et al., 2012). All these basic elements of the RNAi machinery are used by *M. circinelloides* not only to defend the genome against invasive nucleic acids but, more noteworthy, to regulate the expression of their own genes (see below) (Nicolás et al., 2010; Cervantes et al., 2013; Trieu et al., 2015).

M. circinelloides shares with many other fungi the use of this basic RNAi machinery as a defense mechanism against exogenous nucleic acid such as transgenes, viruses and transposons. Small RNA molecules derived from those inducers have been identified in several fungi and demonstrated their protective role from invasive nucleic acids, both during vegetative growth and sexual development. Characteristics of these fungal silencing pathways have been adequately reviewed elsewhere (Dang et al., 2011; Chang et al., 2012; Nicolás and Ruiz-Vázquez, 2013; Billmyre et al., 2013). Significantly, although most fungi contain several *rdrp* genes, an amplification process producing secondary siRNAs similar to that described in *M. circinelloides* (Nicolás et al., 2003) has been only demonstrated in fission yeast (Simmer et al., 2010), whereas *N. crassa*, *Aspergillus* and other fungal RNAi pathways do not include amplification of the silencing signal.

3. Endogenous *dicer*-dependent small RNA molecules of *M. circinelloides*

M. circinelloides accumulates a high amount of esRNAs, which derive from exons of protein coding genes, repetitive sequences and transposons and intergenic regions (Nicolás et al., 2010). Analysis of the distribution of these esRNAs among the different types of loci revealed that they were not formed randomly across the genome, but they were enriched in exonic sequences compared with intergenic and repetitive regions, which is in contrast with other fungi, where most esRNAs are produced from repeats and transposons. These exon-derived esRNAs were named exonic-siRNAs (ex-siRNAs), and comparison of the ex-siRNA content in the wild type strain and RNAi mutants of *M. circinelloides* revealed that a large number of them are *dicer*-dependent, since they required a Dicer enzyme for their production. There are four different classes of *dicer*-dependent ex-siRNAs, depending on the components of the silencing machinery required for their biogenesis (Nicolás et al., 2010; Cervantes et al., 2013) (Fig. 1). Class 2 is the main *dicer*-dependent ex-siRNAs class, and requires Dcl-2 and RdRP-1 proteins for their production. Only a small group of *dcl-2*-dependent ex-siRNAs (class 1) do not require RdRP-1 for their bio-

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