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Carolin Wollschlaeger, Nuria Trevijano-Contador, Xuying Wang, Mélanie Legrand, Oscar Zaragoza, et al.. Distinct and redundant roles of exonucleases in *Cryptococcus neoformans*: Implications for virulence and mating. *Fungal Genetics and Biology*, 2014, 73, pp.20 - 28. 10.1016/j.fgb.2014.09.007 . hal-02636608

**HAL Id: hal-02636608**

**<https://hal.inrae.fr/hal-02636608>**

Submitted on 27 May 2020

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Published in final edited form as:

*Fungal Genet Biol.* 2014 December ; 73: 20–28. doi:10.1016/j.fgb.2014.09.007.

## Distinct and redundant roles of exonucleases in *Cryptococcus neoformans*: Implications for virulence and mating

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### Abstract

Opportunistic pathogens like *Cryptococcus neoformans* are constantly exposed to changing environments, in their natural habitat as well as when encountering a human host. This requires a coordinated program to regulate gene expression that can act at the levels of mRNA synthesis and also mRNA degradation. Here, we find that deletion of the gene encoding the major cytoplasmic 5'→3' exonuclease Xrn1p in *C. neoformans* has important consequences for virulence associated phenotypes such as growth at 37°C, capsule and melanin. In an invertebrate model of cryptococcosis the alteration of these virulence properties corresponds to avirulence of the *xrn1* mutant strains. Additionally, deletion of *XRNI* impairs uni- and bisexual mating. On a molecular level, the absence of *XRNI* is associated with the upregulation of other major exonuclease encoding genes (*i.e.* *XRN2* and *RRP44*). Using inducible alleles of *RRP44* and *XRN2*, we show that artificial overexpression of these genes alters *LAC1* gene expression and mating. Our data thus suggest the existence of a complex interdependent regulation of exonuclease encoding genes that impact upon virulence and mating in *C. neoformans*.

### Keywords

*Cryptococcus neoformans*; virulence; *XRNI*; mating

## 1. Introduction

Common to all messenger RNAs, irrespective of the myriad of functions they fulfil, are their beginning by transcription and their ending by degradation. Whereas transcription occurs exclusively in the nucleus, degradation of mRNAs is a process found in the nucleus as well

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as in the cytoplasm, operated by compartment-specific machineries that are however similar from a mechanistic point of view (see (Garneau et al. 2007) for review). In the nucleus and the cytoplasm, 3'→5' decay is carried out by the exosome (see (Chlebowski et al. 2013) for review). This multiprotein complex harbours two catalytic active subunits, Rrp44p and Rrp6p with the latter being specific to the nucleus. 5'→3' decay requires a protein of the Xrn family (Xrn2p/Rat1p in the nucleus, Xrn1p in the cytoplasm; see (Nagarajan et al. 2013) for review). It could be assumed that mutations in one of these global players have comparable consequences for every transcript and deletions are presumably fatal, which is what is found across species for Rrp44p/Dis3p and Xrn2p/Rat1p. On the other hand, deletion of *XRN1* is tolerated by the cell, yet accompanied by pleiotropic phenotypes, evident from the independent identification of *XRN1* in various screens in *Saccharomyces cerevisiae*. Originally isolated as necessary for nuclear fusion in *S. cerevisiae* (Kim et al. 1990), Xrn1p also regulates a large number of processes including filamentation (Kim and Kim 2002) and resistance to different drugs among which is fluconazole (Kapitzky et al. 2010). Xrn1p was also identified as a regulator of filamentation in *Candida albicans* (An et al. 2004), assigning a potential role for Xrn1p in fungal pathogenesis because of the requirements for filaments in pathogenesis in this species.

Initially the phenotypes of mutants without *XRN1* were attributed exclusively to secondary consequences that the absence of Xrn1p has (*i.e.* alteration of transcript levels). However, it was found in baker's yeast that these phenotypes are in part due to Xrn1p-specific functions that are independent of its exonucleolytic activity (Solinger and Pascolini 1999). Interestingly, Xrn1p has been recently shown to directly associate with chromatin (Haimovich et al. 2013), thus regulating transcription of gene expression.

The basidiomycetous yeast *Cryptococcus neoformans* is a major human pathogen responsible for more than an estimated 1,000,000 infections and about 600,000 deaths per year (Park et al. 2009). Like most fungal pathogens, its global importance is due mainly to its capacity to infect immunocompromised individuals such as HIV/AIDS patients or people receiving organ/bone marrow transplants. Three major virulence factors of *C. neoformans* have been established: 1. the ability to grow at 37°C (Perfect 2006; Vecchiarelli and Monari 2012), 2. the presence of a polysaccharide capsule (Vecchiarelli and Monari 2012) and 3. the production of the pigment melanin (Williamson 1997). The genomes of two varieties of *C. neoformans* have been sequenced and annotated (Loftus et al. 2005; Janbon et al. 2014). These studies revealed very complex transcriptomes being very intron-rich (99% of the genes contain introns) and in which alternative splicing is common (Grützmann et al. 2014; Janbon et al. 2014). Moreover, numbers of long non-coding RNAs (lncRNAs), mainly antisense, have been identified and a large set of proteins orthologous to metazoan serine/arginine-rich (SR) proteins has been identified (Janbon et al. 2014); (Warnecke et al. 2008). *C. neoformans* is an opportunistic pathogen and its natural habitat is outside an animal host *e.g.* in the soil or in association with certain tree species (Lin and Heitman 2006). As such it needs to cope with a large number of stresses. It has been hypothesized that its complex and plastic transcriptome provides an easy way to alter its metabolism in order to colonize successfully a large diversity of environmental niches.

Recently, we identified the two essential exonucleases Xrn2p and Rrp44p as being key partners in the intron-dependent regulation of gene expression in *C. neoformans* (Goebels et al. 2013). Here, we describe the characterisation of a *xrn1* strain in *C. neoformans*. We find that the deletion of this gene is associated with the alteration of several virulence factors which manifests in avirulence of the mutant strain. Further, we find that Xrn1p is needed for the regular succession of the mating process. We also observed that the deletion of *XRN1* is associated with an upregulation of *RRP44* and *XRN2*. Finally, our experiments showed that artificial overexpression of *RRP44* and *XRN2* is sufficient to alter *LAC1* expression and mating. Taken together, these results suggest that a fine-tuned, interdependent regulation of the major exonucleases controls virulence and mating in *C. neoformans*.

## 2. Material and Methods

### 2.1 Strains and culture conditions

*C. neoformans* strains used in this study are all serotype D strains and are listed in Table 1. The strains were routinely cultured on YPD medium at 30°C (Sherman 1992). Synthetic dextrose (SD) was prepared as described (Sherman 1992).

Strains were grown overnight at 30°C in liquid YPD, serially diluted ( $10^4$  –  $10^1$ ) and spotted onto different solid media to determine growth phenotypes. Likewise, melanin production was assessed after spotting serial dilutions of cells of each strain on Niger agar medium (Walton et al. 2005); all plates were read after 48h of incubation at 30°C, unless otherwise stated.

### 2.2 Immunoblotting with monoclonal antibodies (MAb)

The anticapsular MAb E1 (Dromer et al. 1988) (kindly provided by F. Dromer, Institut Pasteur, Paris, France), CRND-8 (Ikeda et al. 1996) (kindly provided by T. Shinoda, Tokyo, Japan), 4H3, 2H1, and 5E4 (Casadevall and Scharff 1991) (kindly provided by A. Casadevall, Albert Einstein College of Medicine, New York, N.Y.) were used in immunoblotting experiments, with techniques as previously described (Janbon et al. 2001).

### 2.3 RNA extraction and northern blot analysis

Cells were routinely harvested after being grown up to  $5 \times 10^7$  cells/mL in YPD. RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Total RNA (5 µg) was separated by denaturing agarose gel electrophoresis and transferred onto Hybond-N+ membrane (GE Healthcare, Piscataway, NJ) and probed with [ $^{32}$ P]dCTP-radiolabelled DNA fragments.  $\beta$ -particle emissions were quantified with a Typhoon 9200 imager (GE Healthcare) and Image Quantifier 5.2 software (Molecular Dynamics, Fairfield, CT).

For the analysis of *LAC1* expression under carbon starvation conditions,  $4 \times 10^6$  cells/mL were grown in 50 mL of YPD or YPG for 3 hours. At 3 hours, cells were collected by centrifugation, washed with sterile distilled water, resuspended in 50 mL of Asparagine medium without glucose (1 g/L asparagine, 0.1 g/L  $\text{MgSO}_4$ , 10 mM  $\text{NaH}_2\text{PO}_4$ ; pH 6.5) and incubated in this medium for another 3 hours.

## 2.4 Gene disruption

The genes described in this report have been deleted by biolistic transformation of a serotype D strain using a disruption cassette constructed by overlapping PCR as previously described (Moyrand et al. 2004). The primer sequences used are given in Table A.1. The transformants were then screened for homologous integration as previously described (Moyrand et al. 2004). The plasmid, pNAT used to amplify the NAT selective marker was kindly provided by Dr Jennifer Lodge (Saint Louis University School of Medicine). The plasmid pPZP-NEO1 was used to amplify the NEO selective marker. Multiple mutant strains were obtained through crosses of single mutant strains on V8 medium as previously described (Moyrand et al. 2004). Progenies were selected on minimum medium. Their genotypes were determined by PCR. The mating types of the strains were determined by crossing them on V8 medium to tester strains of known mating type.

## 2.5 Capsule growth

To induce capsule enlargement, cells were incubated overnight in liquid Sabouraud medium (Oxoid, England) at 30°C with moderate shaking, and then transferred to 10% Sabouraud medium at pH 7.3 buffered with 50 mM MOPS buffer (Zaragoza and Casadevall 2004) for 24 hours. The cells were then suspended in India Ink, and pictures were taken using a Leica DMI3000 microscope (Leica Microsystems, Germany). Capsule size was estimated using Adobe Photoshop 7.0 (Adobe, San Jose, CA). The diameter of the total cell size and of the cell body (delimited by the cell wall) was measured, and capsule size was determined as the difference between these two parameters. Statistically-significant differences between samples were determined using ANOVA and Student's t-Test (significance was considered when  $p < 0.05$ ).

## 2.6 *Galleria mellonella* infection

*Galleria mellonella* were infected as previously described (Mylonakis et al. 2005; García-Rodas et al. 2011). The larvae were obtained from Alcotán (Valencia, Spain). Larvae without any dark spots weighting between 0.2-0.3 g were selected and incubated at 30°C or 37°C the day before the infection. To prepare the inocula, yeast were grown overnight in liquid Sabouraud medium at 30°C with moderate shaking, washed with PBS plus ampicillin (50 µg/mL) and suspended in the same buffer. Cell density was estimated with an automatic cell counter TC10 (Bio-Rad, Hercules, CA). Then, suspensions at  $10^8$  cells/mL were prepared in PBS+ampicillin. Larvae (20 per group) were injected with 10 µL of the yeast suspension ( $10^6$  yeast cells per larva) through the last right proleg, which was previously cleaned with 70% ethanol. A parallel group of larvae was injected with 10 µL of PBS +ampicillin as a control. The larvae were incubated at 30°C or 37°C, and death was daily monitored for 10 days.

Killing curves were adjusted using the Kaplan-Meier method and estimation of differences in survival were analysed with the log rank and Wilcoxon tests using GraphPad Prism 5 software (GraphPad, San Diego, CA). A p-value below 0.05 was considered significant.

## 2.7 *In vivo* phagocytosis assay

Yeasts were grown overnight in liquid Sabouraud medium, washed with PBS and stained with 10 µg/mL Calcofluor white (Sigma, St. Louis, MO) for 30 minutes at 37°C. Then, these cells were injected into *G. mellonella* (10<sup>6</sup> cells/larva) as described above. After 3 hours of incubation at 30°C or 37°C, haemolymph was collected in 1.5 mL tubes in 50 µL cold PBS to avoid coagulation and melanization of the haemolymph. Haemocytes were placed on a slide and phagocytosis was visually quantified using a Leica DMI 3000B. 100 haemocytes from each larva were counted per experiment and results were expressed as the percentage of haemocytes that contained internalized cryptococcal cells.

## 2.8 Growth curves

Yeast strains were grown overnight in liquid Sabouraud medium. Then, cellular suspensions at 2×10<sup>5</sup> cell/mL were prepared in fresh liquid Sabouraud medium and 170 µL were placed in 96-well microdilution plates (Costar, New York, NY). The plate was placed in an IEMS Reader MF spectrophotometer (Thermo Fisher Scientific, Germany) and incubated at 30°C or 37°C with shaking. Optical density (OD) was determined at 540 nm every hour for 72 hours. Data were processed with GraphPad Prism 5 software.

## 2.9 Mating and cell fusion assays

WT and *xrn1* mutant cells of opposite mating type were mixed and co-cultured on 5 % V8 juice agar medium (pH 7) and incubated at room temperature in the dark for 1 week. The colonies were photographed following incubation.

To perform the cell fusion assays, wild-type strains JEC33 (*MATa*) and NE292 (*MATa*), and *xrn1* mutant strains NE809 (*MATa*) and NE867 (*MATa*) were grown in YPD liquid medium overnight. Cells were washed and adjusted to 2×10<sup>7</sup> cells/mL, mixed, and grown on V8 mating medium in the dark for 24 h. The colonies were removed with cell scrapers, resuspended in sterile water, and plated onto YNB medium to select for cell–cell fusion products that have both *LYS2* and *URA5* genes. Plates were incubated at 30°C for 5 days until colonies were observed. The experiment was conducted in three replicates and the average number of colonies was calculated. The fusion efficiency was determined by comparing the average number of the visible colonies from the *xrn1* mutant cross to that from the wild-type cross.

To determine an effect of *xrn1* on same sex mating, the gene was deleted in the hyperfilamentous strain XL280 background (Lin et al. 2005), and filamentation was assessed by incubation on V8 mating medium. Pictures were taken with a Leica stereomicroscope.

## 2.10 Ploidy determination by flow cytometry

Cells were grown to stationary phase in deep well 96-well plates. 10<sup>7</sup> cells were harvested by centrifugation (5 min, 5000 rpm, room temperature) and fixed in 70 % ethanol for 1 hour at 4°C. Cells were then washed with water, resuspended in 200 µL TE buffer and incubated with 0.5 g/L RNaseA (Fermentas, Germany) overnight at 37°C. The next day, cells were centrifuged and resuspended in 500 µL TE buffer. 50 µL of this cell suspension were added



to 450  $\mu$ L Sytox Green (Invitrogen) staining solution (1  $\mu$ M) and flow cytometry was performed on 40,000 cells on the FL1 channel of a MACSQuant flow cytometer (Miltenyi, Germany).

### 3. Results

#### 3.1 Interdependent regulation of the different exonuclease genes

We recently reported reciprocal compensation between the major exonucleases Rrp44p and Xrn2p in *C. neoformans* (Goebels et al. 2013). In the same study, we also found that the deletion of *RRP6* is associated with a slight up-regulation of *RRP44*. We here assayed mRNA accumulation of the major 5'→3' cytoplasmic exonuclease gene *XRNI* in three exonucleolytic mutants. As presented in Figure 1A, expression of *XRNI* is slightly increased in all of them (*rrp44*, *xrn2*, *rrp6*). Moreover, upon deletion of the *XRNI* gene the transcript levels of *RRP44* and *XRNI* are significantly increased (Figure 1B). These data suggest a complex interdependent regulation and possible functional redundancies between the different exonucleases.

#### 3.2 Deletion of *XRNI* results in alteration of multiple virulence factors

We deleted the gene encoding Xrn1p (locus CNE03620) using a nourseothricin marker. The original deletant strain was then backcrossed to a wild-type strain to eliminate the possibility of secondary mutations obscuring the analysis.

As previously reported in *S. cerevisiae* (Larimer and Stevens 1990), the *xrn1* mutants show decreased vegetative growth (Figure 2A). Of particular interest for a human pathogen, in *C. neoformans* this phenotype is exacerbated at 37°C (Figure 2A). Strikingly, we observed an aggravation of the *xrn1*-related growth impairment at 37°C in *MATa* relative to *MATa* strains (Figure 2A, and data not shown). Furthermore, the mutants also show an increased sensitivity to SDS (Figure 2A).

In addition to the ability to grow at 37°C, there are other well-defined virulence factors in *C. neoformans*, such as the presence of an antiphagocytic capsule and the production of melanin. As presented in Figure 2A, *xrn1* cells lack the ability to produce melanin when grown on Niger seed agar. Wild-type *Cryptococcus* strains produce melanin under conditions of glucose starvation mainly via a pathway involving the two copper-oxidases Lac1p and Lac2p, with Lac1p being responsible for the majority of laccase activity (Zhu and Williamson 2004). To test whether the deficiency in melanin production observed in the *xrn1* mutants was a direct consequence of reduced *LACI* expression, we performed northern analyses. The results presented in Figure 3A show that the induction of *LACI* transcription seen in a wild-type strain under glucose-depleted conditions (*i.e.* growth in Asparagine medium) hardly occurs in a *xrn1* mutant.

Arguably the most prominent virulence property of *C. neoformans* is the existence of an antiphagocytic capsule surrounding the yeast cell. Interestingly, an increase in capsule size under capsule inducing conditions (*i.e.* 10% Sabouraud medium) related to the deletion of *XRNI* was found (Figure 2B). The alteration of size was not visible when the cells were cultivated under non-inducing conditions (*i.e.* standard Sabouraud medium). In order to test

whether the deletion of *XRNI* is also accompanied by any structural changes in the polysaccharide capsule, we tested the reactivity of wild-type and mutant cells to different anticapsule antibodies. Although wild-type strains of the D serotype do not normally react with the serotype A-specific antibody E1 (Dromer et al. 1987), we found a low but reproducible reactivity of the *xrn1* strains with this antibody (Figure A.1), pointing to a different structural composition of the capsule (Moyrand et al. 2002) in the absence of *XRNI*. Also, an increased binding affinity of the anticapsule antibody 4H3 (Mukherjee et al. 1992) is seen for strains bearing the *XRNI* deletion (data not shown). In contrast, the same assay using the anticapsular antibodies CRND-8 (Ikeda et al. 1996), 2H1 and 5E4 (Casadevall and Scharff 1991) revealed no difference between the *xrn1* mutant strains and the wild-type.

### 3.3 *xrn1* strains are avirulent in *G. mellonella*

*In vitro* determination of virulence factors can give hints as to whether a certain mutant is attenuated for virulence. However, it is not a sufficient substitute for virulence modelling. To confirm the relevance of the altered virulence properties for the overall virulence of *xrn1* strains, we chose the *G. mellonella* model of cryptococcosis. Besides the general advantages of invertebrate infection models, such as lower costs and ethical concerns, this model also facilitates infection modelling at a temperature of 30°C where the *xrn1* growth deficit is minor (Figure 2A and Figure 4A), allowing the assessment of the virulence of the mutants independently of its overall reduced fitness at elevated (mammalian body) temperature.

In keeping with the physiological (growth) and biochemical (melanin, capsule structure) characteristics found *in vitro*, *xrn1* strains also appeared to be completely avirulent in this invertebrate model of cryptococcosis (Figure 4B). As observed previously, phagocytosis (Figure 4C) does not seem to be an indicator for whether a strain is a successful pathogen or not, but rather a mere consequence of capsule size (García-Rodas et al. 2011) (Figure 2B).

### 3.4 Absence of *XRNI* interferes with mating

The melanin and virulence phenotypes associated with the deletion of *XRNI* were partially reminiscent of the phenotypes reported to be associated with the deletion of the *VAD1* gene (Panepinto et al. 2005). *Vad1p* is the homologue of the *S. cerevisiae* protein Dhh1 which has been shown to activate decapping in response to impaired ribosome elongation thus triggering mRNA to degradation by *Xrn1p* (Fischer and Weis 2002; Sweet et al. 2012). *Vad1p* also negatively regulates mating in *C. neoformans* (Park et al. 2010). Given the role of *Xrn1p* in the regulation of mating in *S. cerevisiae* (Kim et al. 1990; Solinger and Pascolini 1999), we characterised the mating process in the absence of *XRNI* in *C. neoformans*. All possible combinations of crosses, *i.e.* WT(JEC33) x WT(NE292), WT(JEC33) x *xrn1* (NE809), *xrn1* (NE867) x WT(NE292) and *xrn1* (NE867) x *xrn1* (NE809), were tested. Though no obvious mating defect was observed during unilateral mating between mutant and wild-type strains, a significant mating defect with reduced filamentation was apparent in a bilateral mutant cross (Figure 5A). In addition, less basidiospores were generated during the *xrn1* x *xrn1* cross. Furthermore, cell fusion



assays (see 2.9) revealed that *xrn1* cells were less efficient in cell fusion (10 % of wild-type level).

Analysis of the phenotypes of 96 randomly chosen progenies revealed a non-Mendelian repartition of the nourseothricin resistance phenotype (8/96) associated with the *XRN1* deletion cassette (Table A.2). We also analysed the ploidy of these isolates by flow cytometry (see 2.10) but we did not detect any diploid isolates among them (Table 2).

Besides the well-defined  $\alpha$ - $\alpha$  opposite sexual cycle, *C. neoformans* can also undergo  $\alpha$ - $\alpha$  unisexual reproduction. While both these forms of sexual reproduction share common regulators, they are mechanistically similar but distinct (Wang and Lin 2011). To test whether the effect of *XRN1* deletion is specific to only the bisexual cycle, we deleted the gene also in the hypersexual haploid strain XL280 (Lin et al. 2005). As demonstrated in Figure 5B, absence of *XRN1* suppresses the filamentous phenotype of the XL280 strain when grown on mating-inducing media (V8). Based on this observation we conclude that Xrn1p most likely affects part of the regulatory pathway that is common to same and opposite sex mating.

### 3.5 Overexpression of *XRN2* or *RRP44* is enough to alter *LAC1* expression in *C. neoformans*

Our data show that the melanin deficiency phenotype of the *xrn1* strain is probably due to the near absence of *LAC1* expression when the mutant strains are cultured in Asparagine medium. This absence of *LAC1* expression might be due to no expression induction in the *xrn1* mutant. Another possibility is that the *LAC1* transcripts are rapidly degraded in a *xrn1* context due to an increased activity of other exonucleases (Figure 1B and Figure 3A). Using regulatable alleles of *RRP44* (*P<sub>GAL7</sub>::RRP44*) and *XRN2* (*P<sub>GAL7</sub>::XRN2*), where the native promoters have been replaced with the *GAL7* promoter, we assayed *LAC1* transcript levels in these strains growing under inducing conditions (*i.e.* YP Galactose) prior to shifting them to Asparagine medium. The results presented in Figure 3B show that *LAC1* induction is slightly less pronounced in the *P<sub>GAL7</sub>::RRP44* or *P<sub>GAL7</sub>::XRN2* strains compared to wild-type, though clearly superior to the level observed in *xrn1* cells. We also looked at a different way to induce *LAC1* expression by the addition of copper that has been reported for the serotype A (Jiang et al. 2009). However, its independence from the carbon source does not seem to be conserved between serotypes as *LAC1* is almost undetectable in cells grown in Asparagine medium supplemented with glucose irrespective of the addition of copper (data not shown) and thus does not represent an alternative. Overall, although the precise mechanism by which *XRN1* deletion leads to *LAC1* repression remains to be defined, our data demonstrate that the sole overexpression of *RRP44* or *XRN2* is enough to modulate *LAC1* expression.

### 3.6 Induced overexpression of *RRP44* or *XRN2* affects mating

We also tested whether induced overexpression of *RRP44* or *XRN2* would perturb the mating process using the galactose-inducible alleles. Therefore, we co-cultured the different mutant strains together with wild-type or mutant strains on V8 agar supplemented with 2 %

galactose in order to induce transcription from the *GAL7* promoter. Robust filamentation was observed in all possible combinations (data not shown).

To characterise further a potential perturbation of the mating process caused by overexpression of *XRN2* (*XRN2<sup>OE</sup>*) or *RRP44* (*RRP44<sup>OE</sup>*), we screened progeny from different crosses, *i.e.* *RRP44<sup>OE</sup>* x WT, WT x *XRN2<sup>OE</sup>* and *RRP44<sup>OE</sup>* x *XRN2<sup>OE</sup>*. First, by flow cytometrical analysis, we noticed an increased number of diploid strains coming from crosses of the overexpression mutants with WT, *i.e.* *RRP44<sup>OE</sup>* x WT, WT x *XRN2<sup>OE</sup>* (Table 2). Next, we screened the progeny with respect to segregation of different markers (Table A. 2). In these single overexpression mutant x WT crosses, irrespective of a Mendelian-like segregation of the dominant selectable markers used to tag the regulatable alleles (47/96 for *P<sub>GAL7</sub>::RRP44::NAT*, 41/96 for *P<sub>GAL7</sub>::XRN2::NEO*), 15 % of *P<sub>GAL7</sub>::RRP44* and 100 % of *P<sub>GAL7</sub>::XRN2* strains retained the ability to grow on YPD which for haploid strains necessitates a wild-type copy of *RRP44* or *XRN2*, respectively (Goebels et al. 2013). This result suggested that the strains could be heterozygous for these loci. Indeed, PCR analysis of randomly chosen clones confirmed the presence of the additional wild-type allele (Figure 6A and data not shown). Moreover, although detection of potential aneuploidy by multiplex PCR did not yield conclusive results (Figure A.2), a potential aneuploidy for chromosome 4 (harbouring *RRP44* as well as the *MAT* locus) in some of the descendants from the *RRP44<sup>OE</sup>* x WT cross seems likely, as selected *P<sub>GAL7</sub>::RRP44::NAT* strains, that were haploid as assessed by flow cytometry (Figure 6C), filamented when cultured on V8 + 2 % galactose (Figure 6B).

The double mutant cross (*RRP44<sup>OE</sup>* x *XRN2<sup>OE</sup>*) further illustrated the intertwined, yet partially independent roles that these two exonucleases play during the mating process. The more frequent occurrence of diploid progeny, found in the single overexpression mutant x WT crosses (*RRP44<sup>OE</sup>* x WT, WT x *XRN2<sup>OE</sup>*) was also observed in progeny from the *RRP44<sup>OE</sup>* x *XRN2<sup>OE</sup>* cross (Table 2). Further screening of the progenies from this cross yielded an unexpected high portion of *P<sub>GAL7</sub>::RRP44* single mutants (59/96) whereas *P<sub>GAL7</sub>::XRN2* single mutant (16/96), *P<sub>GAL7</sub>::RRP44 P<sub>GAL7</sub>::XRN2* double mutant (11/96) and wild-type (10/96) strains were present in equal, though much lower than expected quantities (Table A.2). As already observed in progeny from the WT x *XRN2<sup>OE</sup>* cross, all *P<sub>GAL7</sub>::XRN2* descendants of the double mutant cross regained the ability to grow on YPD which coincided with the additional presence of the wild-type allele of *XRN2* (Table A.2). Similarly, all 7 double mutant strains that were haploid by flow cytometry contained a wild-type copy of *XRN2* (Table A.2). Overall, these data indicate that increased expression of either of these two exonucleases is sufficient to severely perturb the regular succession of the sexual reproduction process.

## 4. Discussion

This study identifies the cytoplasmic exonuclease Xrn1p as a regulator of multiple virulence factors in *C. neoformans*, extending previous studies that have found a central role for RNA metabolic processes in stress adaptation and virulence in this fungus (see (Bloom and Panepinto 2014) for a recent review).

One consequence of deletion of *XRN1* is severely impaired cryptococcal growth at 37°C. Thermotolerance can be characterised as a virulence factor of “disproportionate importance” (Coelho et al. 2014) as it clearly delimits the comparatively few fungal species capable of causing systemic infections in humans (Robert and Casadevall 2009). Unlike capsule synthesis or melanin production, growth at elevated temperature requires adaptation of the whole cellular machinery and is consequently accompanied by extensive changes in gene expression (Steen et al. 2002; Kraus et al. 2004; Chow et al. 2007). These alterations involve mRNA synthesis as well as degradation. Recent work from the Panepinto lab explored the involvement of Ccr4p in the cellular response to host temperature (Havel et al. 2011; Bloom et al. 2013). Ccr4p-mediated deadenylation represents the initial step in cytoplasmic mRNA turnover that ultimately leads to 5' → 3' degradation by Xrn1p. Partially resembling phenotypes of strains with *CCR4* and *XRN1* mutations underline a functional connection. The heightened importance of Xrn1p in the coordination of mRNA synthesis and degradation was elegantly shown in two global studies in *S. cerevisiae* (Haimovich et al. 2013; Sun et al. 2013). Chromatin association of *S. cerevisiae* Xrn1p as reported by Haimovich et al. points to a role for Xrn1p in transcription. Assuming an involvement of Xrn1p in transcription also in *C. neoformans* could explain the observed impact that *XRN1* deletion has on *LAC1* expression and thus melanin production. Hence, the at first counter-intuitive observation of reduced *LAC1* mRNA levels upon deletion of this exonuclease might be due to an involvement of Xrn1p in *LAC1* transcription alongside with increased degradation of *LAC1* by *xrn1* -induced expression of other exonucleases.

The laccase defect that we found associated with deletion of *XRN1* mimics that of a *vad1* strain. A mutant in the DEAD-box RNA helicase encoding gene *VAD1* was initially isolated in an insertional mutagenesis screen for laccase-deficiency (Panepinto et al. 2005). Localisation studies describe an accumulation of Vad1p in P-body-like structures (Panepinto et al. 2005). These “factories for mRNA decay” are clearly linked to Xrn1p activity in yeast (Sheth and Parker 2003; Kulkarni et al. 2010). A co-operative activity of Vad1p and Xrn1p could thus be at the origin of *LAC1* repression. However, the interaction between Vad1p and Xrn1p does not seem to be readily defined when considering the *xrn1* -related perturbation of the mating process described in this study that is reversed to that found for *vad1* (Park et al. 2010). Further studies are obviously needed to elucidate their interconnection.

The pleiotropic roles of Xrn1p partially stem from its function as an exonuclease. Redundancy of this exonuclease activity in the cell obstructs a clear view of its functions. Comparison of the effect that induced overexpression of *RRP44* and *XRN2* has on the mating process helped to characterise the *XRN1*-related mating phenotype as being distinct from the exonucleolytic compensation, *i.e.* increased expression of *RRP44* and *XRN2* upon *XRN1* deletion. At the same time, our data from mating experiments in which *RRP44* or *XRN2* were overexpressed clearly show the importance of equilibrated levels of exonucleases for successful sexual reproduction. Elucidation of the underlying molecular mechanisms promises to aid in understanding the regulation of the mating process. Overall, the here presented work adds Xrn1p to the growing number of RNA-related factors that orchestrate virulence in *C. neoformans*. It is apparent that a better understanding of the RNA

metabolism present in this basidiomycete can give valuable insight into its multifaceted lifestyle, crucial to counter this pathogenic challenge.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

We are grateful to F. Dromer (France), T. Shinoda (Japan) and A. Casadevall (USA) for their generous gifts of MAb. We thank J. Lodge (USA) for plasmids. This work was supported by a grant from ANR (2010-BLAN-1620-01 program YeastIntrons) to GJ.

CW was a recipient of a scholarship from the Pasteur-Paris University International Doctoral Program/Institut Carnot Maladies Infectieuses.

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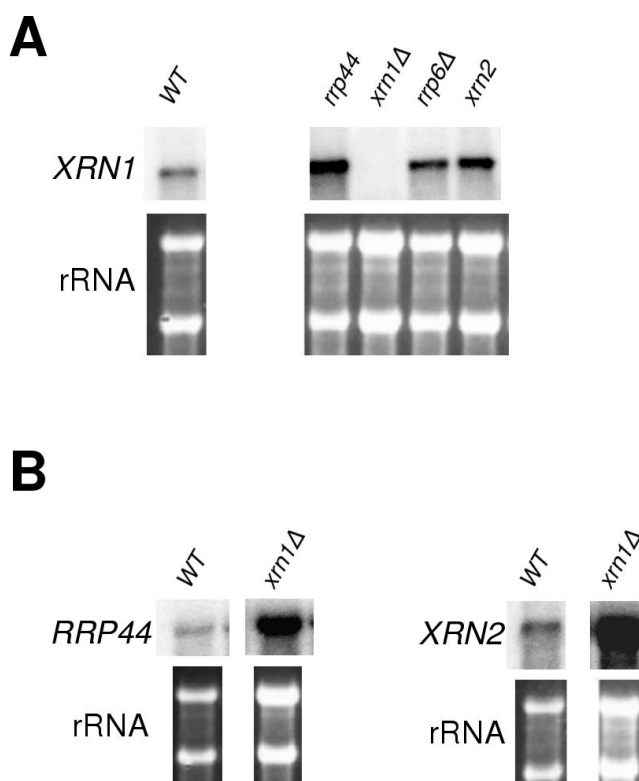
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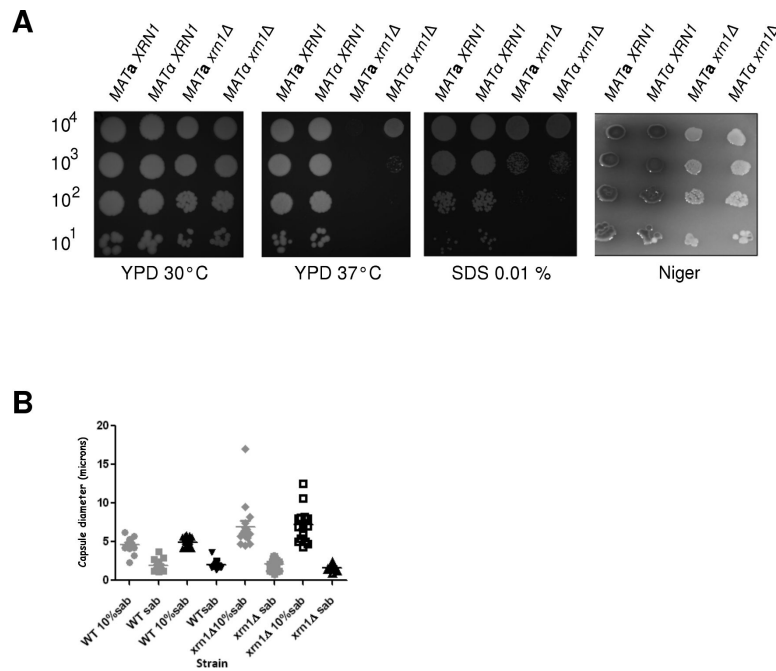


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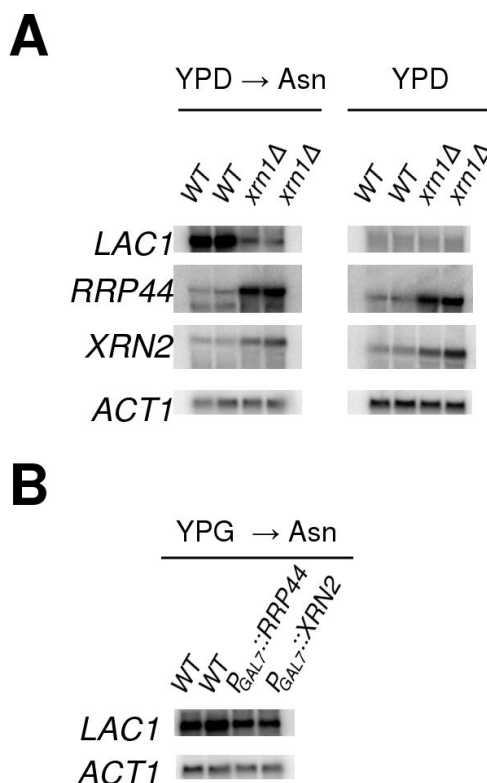
**Figure 1. Widespread compensation between different exonucleases**

**A** Expression of *XRN1* is increased in exonucleolytic mutants. Northern blot experiment results showing the transcript levels of *XRN1* in different exonucleolytic mutants. Relative to wild-type level and normalised to *ACT1*, *XRN1* mRNA abundances are 1.8x in the *rrp44* strain, 1.7x in the *xrn2* strain and 1.3x in the *rrp6Δ* strain. **B** Deletion of *XRN1* is compensated by overexpression of *RRP44* and *XRN2*. Northern blot experiment results showing the transcript levels of *RRP44* and *XRN2* depending on the presence of *XRN1*. Normalised to *ACT1*, *RRP44* and *XRN2* mRNA abundances in the *xrn1Δ* mutant strain are 3.5x and 1.8x respectively, relative to wild-type level.



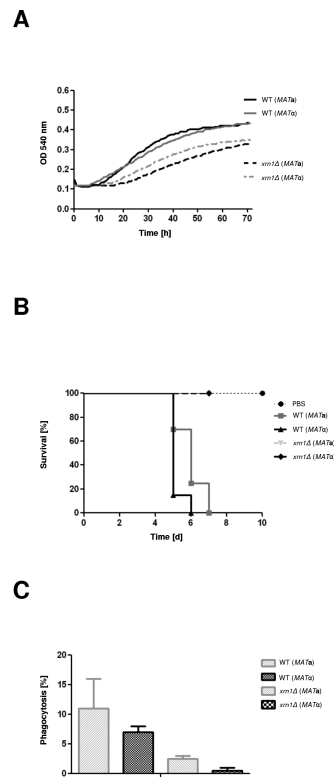
**Figure 2. *XRN1* deletion alters virulence-associated phenotypes**

**A** Growth phenotypes associated with *XRN1* deletion. Serial dilutions of cells were spotted onto different media. Pictures were taken after 4 (YPD) and 5 (Niger) days, respectively. **B** Capsule size differences associated with *XRN1* deletion. Distribution of capsule diameter of wild-type and *xrn1* cells at 30°C in Sabouraud medium and diluted Sabouraud medium. The line in each sample denotes the average of the distribution.



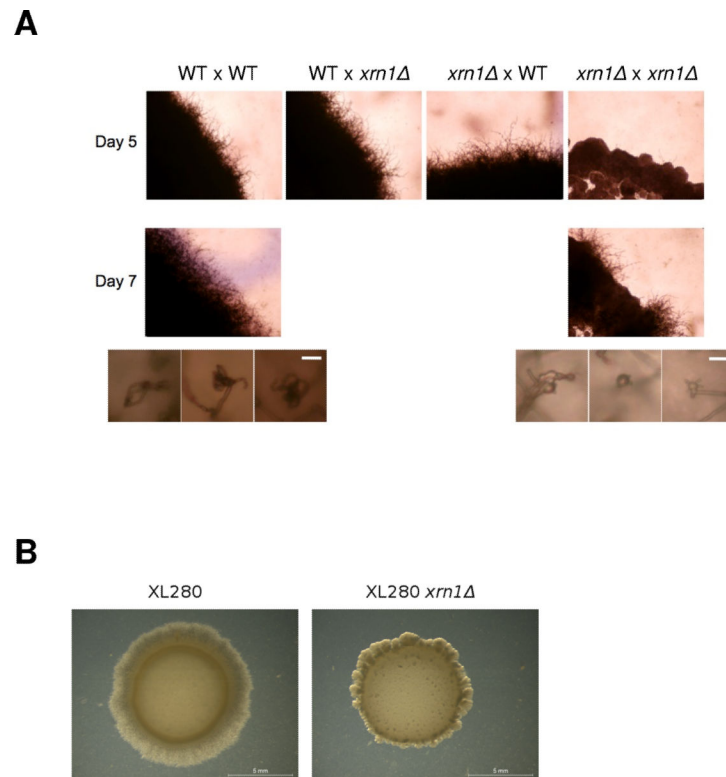
### Figure 3. Expression of *LAC1* requires *XRN1*

Northern blot analysis of RNA from the indicated cells incubated for 3 hours in YPD (A) or YPG (B) and then shifted for an additional 3 hours to Asparagine medium (Asn) or grown for 6 hours in YPD (A) as control. **A** Induction of *LAC1* by glucose starvation (Asn) in WT and *xrn1* cells. Relative to wild-type level and normalised to *ACT1*, *LAC1* mRNA abundance in the *xrn1* strain is 0.1x **B** *LAC1* expression in galactose-inducible mutants of *RRP44* and *XRN2*. Relative to wild-type level and normalised to *ACT1*, *LAC1* mRNA abundances are 0.7x and 0.9x in the *P<sub>GAL7</sub>::XRN2* and *P<sub>GAL7</sub>::RRP44* strains, respectively.



**Figure 4. *xrn1* cells are avirulent**

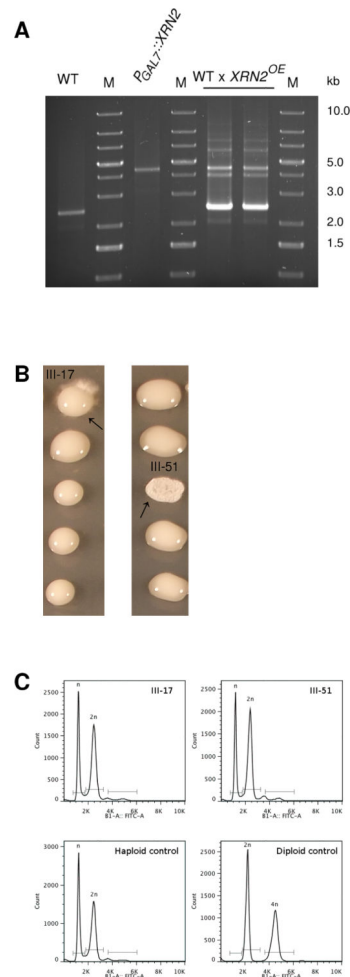
**A** Growth curves of wild-type and *xrn1* cells at 30°C in Sabouraud medium. **B** Survival of *G. mellonella* infected with wild-type and *xrn1* strains at 30°C. **C** *In vivo* phagocytosis of *G. mellonella* larvae infected with wild-type and *xrn1* cells at 30°C.



### Figure 5. Deletion of *XRN1* affects sexual reproduction

**A** *xrn1* mutants show a defect in bisexual mating. Mating between WT and *xrn1* mutant cells of opposite mating type were mixed and co-cultured on 5% V8 juice agar medium (pH 7) and incubated at room temperature in the dark for 1 week. The colonies were photographed following incubation. Scale bar = 10 μm. **B** Deletion of *XRN1* suppresses the hyperfilamentous phenotype of XL280. Strains were incubated on V8 juice agar medium (pH 7) for 15 days at room temperature in the dark. Pictures were taken with a Leica stereomicroscope. Scale bar = 5 mm.





**Figure 6. Overexpression of *RRP44* or *XRN2* perturbs mating**

**A** Matings in which *XRN2* is overexpressed (WT x *XRN2*<sup>OE</sup>) yield haploid progeny that contain both, the wild-type *XRN2* and the *P*<sub>GAL7</sub>::*XRN2* alleles. PCR analysis showing the simultaneous presence of wild-type and *P*<sub>GAL7</sub> alleles of *XRN2* in progeny from the WT x *XRN2*<sup>OE</sup> cross. PCR with primers flanking the *XRN2* promoter region yield a fragment of about 2.7 kb for the wild-type allele and 4.5 kb for the *P*<sub>GAL7</sub> allele. **B** Progeny strains (III-17, III-51) from matings in which *RRP44* is overexpressed (*RRP44*<sup>OE</sup> x WT) filament on V8 + 2 % galactose. **C** Progeny strains (III-17, III-51) from matings in which *RRP44* is overexpressed (*RRP44*<sup>OE</sup> x WT) are haploid as determined by flow cytometry.

Table 1

List of the *C. neoformans* var. *neoformans* strains used in this study.

Name	Genotype	Construction details	Reference
JEC33	<i>MATa lys2</i>		Wickes and Edman, 1995
XL280	<i>MATa</i>		Lin et al., 2005
NE292	<i>MATa cas3 i ade2 ura5</i>		Goebels et al., 2013
NE718	<i>MATa rrp6 ::NEO cas3 i ura5</i>		Goebels et al., 2013
NE730	<i>MATa NAT-P<sub>GAL7</sub>::RRP44 lys2</i>		Goebels et al., 2013
NE787	<i>MATa NAT-P<sub>GAL7</sub>::RRP44</i>		Goebels et al., 2013
NE791	<i>MATa cas3 i NAT-P<sub>GAL7</sub>::RRP44</i>		Goebels et al., 2013
NE809	<i>MATa xml1 ::NAT cas3 i ade2 ura5</i>	Biolistic transformation NE292	This study
NE822	<i>MAT a</i>	Cross JEC33 × NE809	This study
NE824	<i>MATa</i>	Cross JEC33 × NE809	This study
NE826	<i>MATa xml1 ::NAT</i>	Cross JEC33 × NE809	This study
NE828	<i>MATa xml1 ::NAT</i>	Cross JEC33 × NE809	This study
NE867	<i>MATa xml1 ::NEO lys2</i>	Biolistic transformation JEC33	This study
NE856	<i>MATa NEO-P<sub>GAL7</sub>::XRN2 lys2</i>		Goebels et al., 2013
NE874	<i>MATa cas3 i NEO-P<sub>GAL7</sub>::XRN2 ade2 ura5</i>		Goebels et al., 2013
NE923	<i>MATa xml1 ::NAT</i>	Biolistic transformation XL280	This study
NE924	<i>MATa xml1 ::NEO</i>	Biolistic transformation XL280	This study

Table 2

Results of ploidy analysis by flow cytometry.

Cross	Number of isolates analysed	Number of diploid strains
WT × WT	86	1
WT × <i>xml</i>	92	0
<i>RRP44<sup>OE</sup></i> × WT	92	4
WT × <i>XRN2<sup>OE</sup></i>	93	3
<i>RRP44<sup>OE</sup></i> × <i>XRN2<sup>OE</sup></i>	94	6