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A novel phenotype of eight spores asci in deletants of the prion-like Rnq1p in *Saccharomyces cerevisiae*

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Abstract

We report that a null *rnq1* mutation in the yeast *RNQ1* (YCL028w) prion-like gene of so far unknown function produces the doubling of spores in the asci. This phenotype is possibly due to the lack of inhibition by Rnq1p of an additional mitotic division during ascus formation. This novel phenotype termed "octopus asci" could be similar to prion [PIN+] phenotype.

Keywords: *Saccharomyces cerevisiae*; RNQ1; Yeast prion; Octopus asci

The sequencing of 16 chromosomes of *Saccharomyces cerevisiae* was completed in 1997. Today, the number of protein-coding genes is estimated to be 5800. The functions of over one thousand remain undefined. One of them is the YCL28w gene on chromosome III [1,2].

The ORF YCL28w was described over 15 years ago as encoding a protein "rich in asparagine and glutamine" and therefore termed Rnq1p [6]. This feature is related to the two genetically best-characterized yeast prion gene products, Sup35p and Ure2p, that have polyglutamine-rich amino-terminal domains known to be critical for prion formation and maintenance [7–9]. However, the Rnq1p asparagine and glutamine domain is located at its carboxyl terminus [6]. Propagation of [URE3] and [PSI+] is dependent on the continuous expression of the respectively associated gene products Ure2p and Sup35p that are necessary for normal cells. Ure2p inhibits the transcriptional regulator Gln3p, which is required for activation of genes involved in nitrogen catabolism. Sup35p is a subunit of the translation termination factor complex [10–13]. The phenotypes of ure2 and sup35 loss of function mutants are identical with those of cells bearing prion [7,12,13].

As for several other yeast prions [6,14], Rnq1p aggregates in certain strains and is soluble in other. The aggregated state is dominant and can be transmitted by cytoduction [14,15]. Prion Rnq1p is cured by growth in the presence of guanidine hydrochloride. Polyglutamine (polyQ) expansions in several human genes coding unrelated proteins cause their aggregation and are responsible for at least eight inherited neurodegenerative diseases [16,17].

Rnq1p is required for Sup35p prion [PSI+] formation de novo [14,15] and therefore termed [PIN+]. However, Rnq1p is not necessary for the propagation of [PSI+] [18,19]. Furthermore, spontaneous appearance of [PIN+] is accompanied by the appearance of Rnq1p aggregates. Sondheimer and Lindquist [6] have reported that the prion-like domain of Rnq1p can be transferred to another protein to produce an epigenetic modification. Therefore, *RNQ1* was claimed to be a new yeast prion [7,8].

The only phenotype diagnostic for cells carrying the prion form of Rnq1p is the increased yield of de novo formation of other yeast prion [PSI+] [6,8]. This phenotype is influenced by the strongly interacting [PIN+] and [PSI+] prions [7,18,19].

Under sporulation conditions the wild diploid cells are converted into asci containing four meiotic products termed ascospores. Several mutants with abnormal spore formation have been reported such as the classic *spo*

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mutants. Some mutants sporulate normally at 28 °C and form no asci at higher temperatures. Other sporulation-defective mutants have been revealed by genetic analysis of strains bearing mutations in genes that, a priori, are not expected to be required for sporulation. There are also mutants which produce asci with only two or three viable spores [5,20].

The aim of our work was to find whether it is possible to estimate numerically the changes during the formation of zygotes and tetrads in strains disrupted in *RNQ1*.

Materials and methods

Strains, plasmids, and media. *Saccharomyces cerevisiae* strains and plasmids are listed in Table 1. Standard yeast media were prepared according to Sherman [21]. Standard DNA manipulation methods are described by Sambrook et al. [22].

Disruption of YCR028 (RNQ1) gene. The RNW303 null RNQ1 mutation was obtained by gene replacement in haploid strains with a loxP-kanMX-loxP cassette flanked by short regions homologous to the target gene [26]. Disruption cassette for RNQ1 was obtained by PCR using the pUG6 plasmid as a template [24] with the following primers: 5′-CCA TgC AgA AgC TgT TgC gAA gTT gAC ATC CgC AgC TCA gTg CCg CTg AAg CTT CgT ACg CT-3′ (forward) and 5′-gCT gTC CAT TTg gAg TTC TggTTT CCg CCg TAT TgC GGT CTg CAT Agg CCA CTA gTg gAT CTg-3′ (reverse). Null mutants were obtained according to Baudin et al. [27]. All disruptions were confirmed by PCR amplification of the novel DNA junction.

Construction of cure plasmid. The cure plasmid was based on pFL 44s in which gene *RNQ1* was inserted using *Bam*HI/*Pst*I restriction sites.

Homozygous diploid strain. Target gene deletions were performed in the diploid strains W303. Heterozygous diploid mutants were dropped on sporulating medium [21]. The asci were dissected and the spores were tested for the presence of the kan-cassette and for mating type. To obtain diploid homozygous null *rnq1* mutants, haploid strains with opposite mating type were crossed. As the diploid strain cannot be selected the zygotes were isolated from the mating mixture with a micromanipulator. Mating abilities were tested by mixing the same volume of fresh cultures and synchronization according to Jacob's procedure [28].

Yield of sporulation. To identify the diploid homozygous mutants, the zygotes were isolated and tetrad analysis was performed with the de Fonbrune micromanipulator [29]. Zygote formation and sporulation yield were estimated by counting at least 400 cells in Malassez haemocytometer.

DAPI staining. Yeast cells were fixed overnight in 70% ethanol, washed with H_2O, stained with 1 μg/ml DAPI for 10 min, and observed under Nomarski optics and then by fluorescence microscopy using appropriate filters.

Scanning electron microscopy. The final pellets of cells were resuspended and fixed for 2 h at room temperature with 2.5% glutaraldehyde in PBS buffer, pH 7.4, and washed in PBS. The material was postfixed for 60 min with 1% osmium tetroxide in PBS at 4 °C and thoroughly washed doubly in distilled water. Microscope slide was covered with a droplet of yeast suspension. Cells attached to the coverslip were dehydrated with a series of ascending concentrations from 50% to 100% alcohol and air-dried. The slides were mounted on metal stubs and sputter-coated with silver in the high-vacuum sputtering apparatus (Hochvakuum Dresden B-31). Samples were examined in a Tesla BS 300 scanning electron microscope at an accelerating voltage of 20 kV.

Results

After 4-day incubation of the strain RNW303D on the sporulation medium, viable asci with more than four spores were observed among normal tetrads.

The diploid homozygous strain RNW303D was obtained by crossing the isogenic strains RNW303B and RNW303A that differ only in the MAT gene. Under typical selective condition [3,4] the tested diploid strains produced about 26% of zygotes—comparable to the corresponding strain W303. The mutant strain RNW303D formed tetrads with 42% efficiency which is higher than that of the wild-type strain (average of 10 experiments). Atypical asci with more than four asci were observed at a low frequency among 50 independent diploid strains RNW303D.

The number of atypical asci fluctuated between 1% and 4% total asci (average 1.8%). The highest efficiencies were obtained when the diploid clones were pre-incubated on pre-sporulating liquid medium and then incubated on solid sporulating medium (Fig. 1).

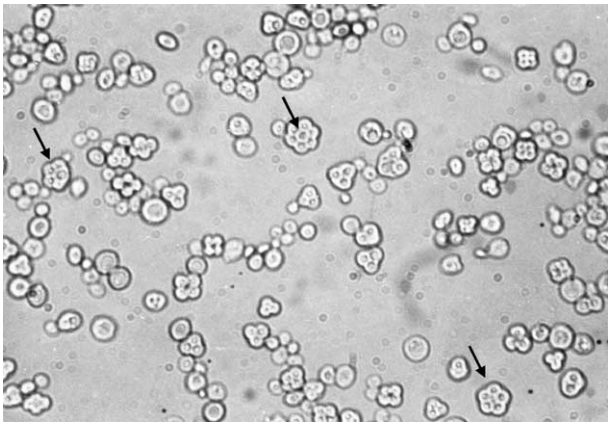

Fig. 1. Tetrads and atypical asci (marked with arrows) in Rnq1 diploid strain viewed under light optic microscope. Magnification 400×.

Table 1
Yeast strains and plasmids used in the work

Strain/plasmid	Genotype	Source
W303	MATα/MATa ura3-1/ura3-1, trp1-1/trp1-1, ade2-1/ade2-1, leu2-3,112/leu2-3,112, his3-11,15/ his3-11,15	[23]
RNW303	W303; *YCR028*/ycr028Δ:: loxP-kanMX-loxP	This work
RNW303B	MATα ura3-1, trp1-1, ade2-1, leu2-3,112, his3-11,15 ycr028Δ:: loxP-kanMX-loxP	This work
RNW303A	MAT**a** ura3-1, trp1-1, ade2-1, leu2-3,112, his3-11,15 ycr028Δ:: loxP-kanMX-loxP	This work
RNW303D	Homozygous diploid	This work
pUG6	Vector containing the *loxP-kan-loxP* deletion cassette	[24]
pFL44S	Multicopy vector, Amp^R, URA3	[25]
pFL44-RNQ	pLF44S with the insert *RNQ1*	This work

Not all of the separated spores could germinate. The lacking spores were not lost during manipulations, since non-germinative spores were observed on the agar slide under light microscope. Table 2 shows that multisporal asci contain up to eight ascospores. Under light microscope six or seven spores asci were observed frequently, but among viable spores "hidden" spores could be present. Images from scanning microscope confirmed the light-microscopic observation that all eight spores have a common cell wall (Fig. 2).

In the population of atypical tetrads, the most common asci comprised only 5–7 spores. The DAPI-stained nuclei were observed to assess whether all ascospores originating from atypical asci possess their own nuclei. As illustrated in Fig. 3, abnormal asci show an increased amount of nuclei (from 5 to 8) in good agreement with the observation of spores under light microscope.

The distribution of the mating type of spores derived from single asci is shown in Table 3, a large variation of mating type segregation observed with one particularly interesting segregation of 5 MAT**α**:1 MAT**a**.

Table 2
Germination of spores derived from single atypical ascus

Total number of spores per asci	Number of germinated spores per analysed asci
5	Five germinated spores in three asci
6	Six germinated spores in two asci Five germinated spores in four asci
7	Six germinated spores in six asci Five germinated spores in two asci
8	Eight germinated spores in one ascus

Spores were germinated on agar slice put on complete medium plate at 27 °C. Some acsi could not be isolated or analysed.

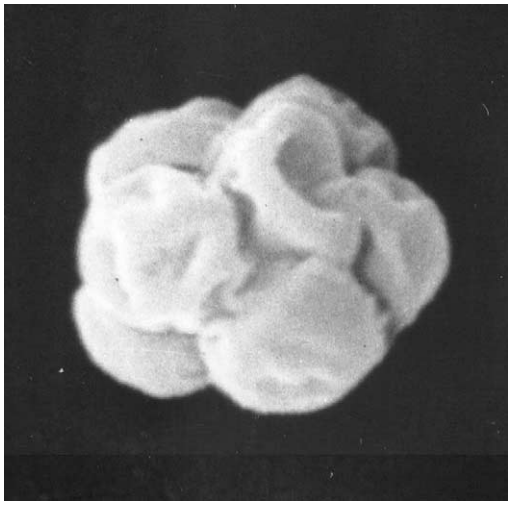

Fig. 2. A typical ascus of RNW303D strain with eight ascospores inside a common cell wall (the last is one on the black side of the moon). Scanning microscope, magnification 6500×.

Discussion

Two possible events could explain the octopus asci.

(1) During spore formation haploid ascospores could redivide mitotically as in *Neurospora crassa*. The *RNQ1* gene product could prevent the cell cycle exit. Therefore, the spore formation process is not complete and some extra mitotic division could take place independently in each spore. This would explain the variable amount of ascospores in untypical asci.

(2) As a consequence of wrong nucleus distribution during the first mitosis preceding conjugation, one of the haploid partners could contain two nuclei and conjugate with a normal haploid. This is unlikely as DAPI staining of haploid "a" and "α" mating cells and of diploid cells did not indicate the presence of additional nuclei.

The asymmetric distribution of Mata or Matα type spores in single asci (Table 3) suggests that additional

Table 3
Mating type of spores derived from single asci

Number of spores in ascus	MATα	MATa
5	2	3
5	3	2
6	4	2
6	5	1
6	2	4
7	4	3
8	4	4

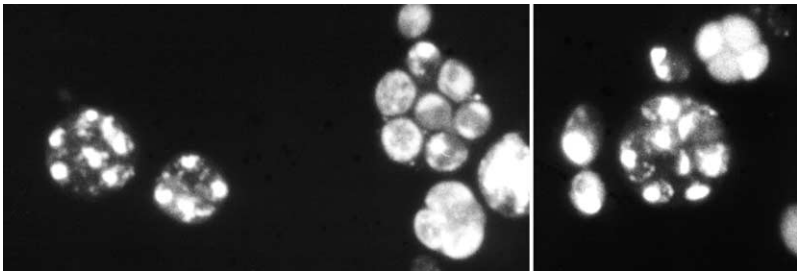

Fig. 3. Localization of nuclei in ascospores of strain RNW303D (DAPI dyed cultures), magnification 1000×.

mitotic division occurs independently in each spore. In particular, the segregation ratio 5:1 confirms this possibility as well as the observation of an ascus having typical Mendelian segregation 4a:4α.

Over-expression of the wild-type copy of the *RNQ1* gene causes the formation of multisporal asci at an extremely low frequency. This fulfils two criteria for recognizing a given protein as a prion. Indeed, prion formation might be induced by over-expression of the normal active Rnq1p which would switch to an inactive form inhibiting a physiological protein involved in spore formation.

Moreover, the low frequencies of multisporal asci suggest that Rnq1p is one, but not the only, inhibitory element of the spore-formation pathway.

In summary, we have observed a novel yeast phenotype which is a rare frequency of octopus asci induced by the RNQ1 null mutation possibly due to an additional mitotic division during sporulation that is normally inhibited by Rnq1p.

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