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YOR129c, a new element interacting with Cnm67p, a component of the spindle pole body of *Saccharomyces cerevisiae*[✪]

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The *Saccharomyces cerevisiae* spindle pole body (SPB) consists of numerous proteins forming the outer, inner and central plaques. The protein Cnm67 is an important component of the outer plaque. The C-terminus of this protein contains a determinant important for its SPB localization. We identified a protein encoded by *YOR129c* which interacts with this C-terminus in the two-hybrid system. *YOR129c* and *CNM67* exhibit weak genetic interaction. The double deletion strain *yor129c*Δ *cnm67*Δ exhibits moderately increased resistance to 0.1 M LiCl and hygromycin B compared with the *cnm67*Δ single mutant. We propose that the YOR129c protein is an accessory factor associated with the cytoplasmic face of SPB and plays a role in cation homeostasis and/or multidrug resistance.

The yeast spindle pole body (SPB) is the functional equivalent of the centrosome of animal cells and organizes microtubules by forming two poles of the mitotic spindle. The SPB is a multilaminar cylindrical structure and is anchored in the nuclear envelope *via* elements that project from its central plaque. The outer plaque of this organellum faces the cytoplasm, and the inner plaque is directed to the nucleus. Since there is no disassembly of the yeast nucleus during mitosis, the localization of the inner and outer plaques in relation to the nuclear envelope remains unchanged during the cell cycle (for a recent review see

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; SPB, spindle pole body.

Helfant, 2002). There are data suggesting that the outer plaque and some part of the central plaque are built from cytoplasmic components, whereas the inner plaque is assembled in the nucleus (Kilmartin & Goh, 1996; Knop & Schiebel, 1998)

The spindle pole body is composed of at least 18 elements that have been identified by various genetic and molecular biology approaches. These proteins can be classed into a few groups. The first one comprises proteins involved in the assembly and maintenance of the mitotic spindle (Stu1, Stu2, Spc19, Spc34, Spc72, Spc97, Spc98, Spc110, Tub4, Bbp1), the second – proteins required for nuclear division and nucleus migration (Cmd1, Cnm67, Nud1, Spc72 (also a member of the first group). The third group is formed by Cdc31, Kar1, and Spc29 proteins, involved in SPB duplication. In addition, there are two outgroup proteins: Ndc1p involved in SPB nuclear envelope insertion (Chial *et al.*, 1999), and the regulatory protein Cmd1 encoding calmodulin, the major calcium sensor, involved also in the nuclear division and the cell cycle (Ohya & Botstein, 1994; Rasmussen & Rasmussen, 1995).

In this report we describe a protein encoded by the open reading frame *YOR129c*. This gene was isolated during a search for suppressors of defects in the heme biosynthetic pathway. Further analysis failed to confirm its role in this process, but, surprisingly, we found that *YOR129c* product interacts with Cnm67p, an important component of the spindle pole body.

MATERIALS AND METHODS

Strains, media and general methods. The *S. cerevisiae* strains are listed in Table 1. The *Escherichia coli* strain was DH5α (Hanahan, 1983). Yeast cells were grown in complete (YPD:1% yeast extract, 1% Bacto-peptone, 2% glucose) or minimal media (0.67% Yeast Nitrogen Base without amino acids, supplemented with 2% carbon source and appropriate amino acids and nucleotides). For the selection of geneticin-resistant transformants, cells were grown on YPD plates containing 400 mg/l G-418 (Roche). Standard procedures for crosses, sporulation, spore dissection and analysis were used (Rose *et al.*, 1990). For phenotypic

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source
BY4742	MATα *; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*	Euroscarf
BY4742*YOR129cΔ*	MATα *; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0 YOR129c::kanMX4*	Euroscarf
BY4741*YNL225cΔ*	MAT*a his3Δ1 leu2Δ0 LYS2 met15Δ0 ura3Δ0 YNL225c::kanMX4*	Euroscarf
MWD1	MAT*a*/α *his3Δ1/ his3Δ1 leu2Δ0/ leu2Δ0 LYS2/lys2Δ0MET15/met15Δ0 ura3Δ0/ura3Δ0 YOR129c::kanMX4/YOR129c YNL225c::kanMX4*	This study
MWD1/1D	MAT*a his3Δ1 leu2Δ0 lys2Δ0 MET ura3Δ0 YNL225c::kanMX4 YOR129c::kanMX4*	This study

tests overnight YPD cultures were serially diluted in growth medium and 5 μl aliquots of 10^{-1}, 10^{-2}, 10^{-3} dilution were spotted onto solid media and grown for 2–4 days at 16°C, 30°C, and 37°C.

Plasmids, DNA manipulation and Western blotting. The plasmids pBluescript II KS (Stratagene) and pRS315 (Sikorski & Hieter, 1989) were used to clone desired fragments of the ORF under study. The plasmid pUG35 C-FUS, obtained from J.H. Hegemann (Inst. Mikrobiol., Dusseldorf, Germany), is a derivative of pGFP-C-FUS (Niedenthal *et al.*, 1996) and contains a GFP-gene version with enhanced fluorescence properties (Cormack *et al.*, 1997). Hemagglutinin (HA) triple epitope was PCR-amplified from an HA-bearing KS vector (Tyers *et al.*, 1993). Standard techniques were used for DNA manipulation and Western blot analysis (Sambrook & Russel, 2001).

Two-hybrid and GFP fusions, HA-epitope introduction. *YOR129c* was PCR-amplified on genomic DNA using the following primers: 5′ GGAATTCATGTTGCGAAGAGAACTA 3′ (introducing *Eco*RI restriction site, underlined, before the ATG codon) and 5′ CGGTTCGTAGCCATGCAACA 3′. The PCR amplified fragment was cloned into the *Eco*RI and *Hin*dIII restriction sites of pBluescript to give the plasmid pMAP20/1. Triple HA epitope was introduced into the *Bsm*I site (position 3616 of the nucleotide sequence) localized in the C-terminal part of the YOR129c-encoded protein, giving the plasmid pUM13.

Two plasmids bearing YOR129c-GFP C-terminal fusion were constructed: pUM15 bearing the fusion under the *MET25* promoter and pUM25 with the same fusion under the original YOR129c promoter. The plasmid pUM15 was constructed by amplifying *YOR129c* using primers: 5′ GGAATTCATGTTGCGAAGAGAACTA 3′ and 5′ CCCAAGCTTGGGCTTTTGAGTGGTTTCTAA 3′, introducing *Eco*RI and *Hin*dIII restriction sites (underlined). The resulting *Eco*RI–*Hin*dIII fragment was cloned into pUG35. The plasmid pUM25 was constructed by amplifying *YOR129c* together with its original promoter using primers: 5′ TATAAGCCTCACCAATGCGG 3′ and 5′ CGGTTCGTAGCCATGCAACA 3′. The resulting 3.7 kb fragment was Klenow-blunted and inserted into pBluescript KS digested with *Not*I and *Pst*I and blunted. From this plasmid the 2.5 kb *Eco*RI fragment, blunt ended with Klenow polymerase and digested again with *Bgl*II, was cloned into pUM15 (digested with *Ecl*136II and *Bgl*II) to yield pUM25.

For two-hybrid interactions the plasmid pMAP20/1 (described above) was digested with *Eco*RI and *Sal*I (present in pBluescript multicloning site) and the resulting 2.7 kb fragment was ligated into *Eco*RI- and *Sal*I-cleaved pBTM116 and pGAD424 plasmids to give pBM1 and pGM1, respectively. All PCR products used in the above constructs were sequenced to exclude errors.

Yeast two-hybrid screen strategy. The two-hybrid studies were done according to the protocols described by Vojtek *et al.* (1997), by sequential transformation using the genomic library of Fromont-Racine *et al.* (1997). Direct two-hybrid analyses were done with cotransformed haploid cells. The host strain for the two-hybrid studies was L40 (Hollenberg *et al.*, 1995). Filter lifts, assays of β-galactosidase activity and growth on $Leu^-Trp^-His^-$ plates were done as recommended by the above protocols. Growth on media lacking histidine was tested in the presence of 50 and 60 mM 3-amino- 1,2,4-triazole. To eliminate false positives, the library isolates were screened against LexA-ras and LexA-erg20 as a bait.

Microscopy. For fluorescence microscopy cells expressing the GFP-tagged protein were stained *in vivo* with DAPI added directly to the growth medium to about 4 μg/ml and incubated for 2–3 h. Cells were subsequently spun down, rinsed twice with water and viewed under a Nikon Microphot-SA microscope. Cells were photographed on Kodak Ektachrome P1600.

RESULTS AND DISCUSSION

The *YOR129c*-encoded protein can be localized to cellular membranes

The open reading frame *YOR129c* encodes an 893 amino acid-long protein of deduced amino-acid sequence showing no significant homology to any known protein. The analysis of this protein started from establishing its cellular localization in a hope to obtain a clue concerning its function. The localization of the *YOR129c* product predicted by PSORT (http://psort.nibb.ac.jp/form2.html) indicated that this protein can be nuclear (56% probability) even though there are no classical nuclear localization signals present in its amino-acid sequence. To verify this prediction we initially constructed a *YOR129c-GFP* fusion gene expressed under the control of the original *YOR129c* promoter. The product of the chimeric gene was undetectable under the fluorescence microscope probably because of poor expression of the gene. However, when the fusion gene was expressed from the regulatory *MET25* promoter, the product was easily detected. Figure 1 shows that the fusion protein YOR129c-GFP is present in the region of the cell nucleus, and a part of it accumulates in the vicinity of the plasma membrane. The weak fluorescence of the whole cells probably results from the plasma membrane. However, such an expression pattern may also suggest that YOR129c protein is not necessarily localized to the inside of the nucleus but may also reside in the endoplasmic reticulum since large parts of the endoplasmic reticulum of *S. cerevisiae* are located close to intracellular organelles (Pichler *et al.*, 2001). We observed that the localization pattern does not depend on the cell cycle and is the same in budding and non-budding cells. To exclude the posibility that a fraction of the YOR129c-encoded protein is localized in the cell wall, cells were digested with Zymolyase 100T in an isotonic medium, washed and examined under the fluorescence microscope. Spheroplasts bearing the fusion protein retained the pattern of fluorescence presented in Fig. 1. To exclude the possibility that the observed localization resulted from the strong overexpression of the fusion gene leading to an excess of the protein being erratically directed to other than native

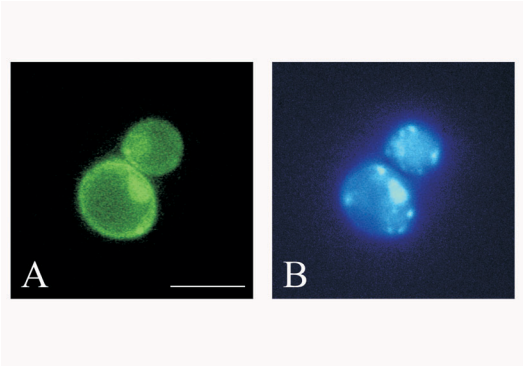

Figure 1. Subcellular localization of the YOR129c-GFP fusion.

The *yor129c*Δ strain was transformed with plasmid pUM15 encoding the above fusion. GFP (A) and DAPI (B) were visualized by fluorescence microscopy. Bar, 10 μm.

compartment(s) we grew yeast bearing the fusion in media containing increasing amounts of methionine (up to 500 μM) to repress the transcription from the *MET25* promoter, but the fusion protein, although produced in decreasing amounts, retained its localization.

The *YOR129c* product is present preferentially in intensely dividing cells

In the next experiment we compared the level of the *YOR129c*-encoded protein in the logarithmic and stationary growth phases. This time the gene was expressed from the original promoter and the GFP tag was replaced with triple HA epitope fused to the C-terminus. This prevented the possible stabilization of the chimeric protein by the proteolysis-resistant GFP. The level of the YOR129c-HA chimeric protein was assayed in cells grown in minimal medium with 2% glucose or ethanol as a carbon source. The culture was inoculated with cells from the late stationary growth phase to the absorbance equivalent to

the early logarithmic phase (1×10^6 cells/ml). Samples were taken after 4, 8 and 24 h after inoculation (densities 3×10^6, 1×10^7, 1×10^8 cells/ml, respectively). A quantitative immunoblot of these samples is presented in Fig. 2. The YOR129c product is most abundant in the vigorously dividing cells and significantly decreases in the stationary phase.

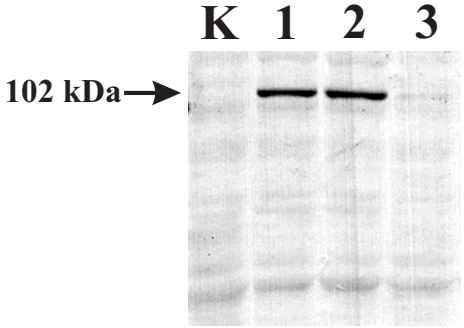

Figure 2. Expression of the YOR129c protein in the logarithmic and stationary growth phases.

The *yor129c*Δ strain was transformed with plasmid pUM13 bearing YOR129c-HA fusion expressed from the original promoter. Equal amounts of protein, derived from 2×10^6 cells, were loaded on each lane. Total protein was extracted and analyzed by immunoblotting using anti-HA specific antibody. Arrow indicates the YOR129c protein. K, control culture at 3×10^6 cells/ml of the strain transformed with plasmid without fusion, lane 1, culture at 3×10^6, lane 2, 1×10^7, lane 3, 1×10^8 cells/ml.

The YOR129c protein interacts with the C-terminal fragment of Cnm67p in the two-hybrid assay

To clarify the role of the YOR129c protein we performed a two-hybrid assay using this protein as a bait (plasmid pBM1). The screen was performed in a stringent manner (50 mM aminotriazole) and about 900 000 transformants were tested (four independent transformations). In this assay we identified 13 times an identical C-terminal fragment of the Cnm67 protein. This fragment comprises the region starting from lysine 406 through the stop codon at position 581 and constitutes about 30% of the whole Cnm67p.

Cnm67 is a component of the outer plaque of the spindle pole body (SPB), the yeast microtubule-organizing center (Schaerer *et al.*, 2001). It has been shown that the loss of Cnm67 results in the loss of the SPB outer plaque, and this impairs the formation of astral microtubules at this substructure, without altering the dynamics of the spindle microtubules or the formation of astral microtubules at the half-bridge (Brachat *et al.*, 1998; Hoepfner *et al.*, 2000). There was no other localization reported for native Cnm67p apart from SPB. Using partial deletion mutants Schaerer *et al.* (2001) showed that a determinant important for the SPB localization of Cnm67p is localized in the C-terminus of this protein. Cnm67p devoid of the C-terminus (amino acids deleted from position 448) was unable to localize to the SPB and, as a consequence, the outer plaque was not formed. The C-terminal fragment of the protein by itself was not sufficient to localize to the SPB.

The very high number of identical two-hybrid analysis isolates indicates that the YOR129c protein interacts with the C-terminus of Cnm67p, the domain responsible for the SPB localization of this protein. Another protein strongly interacting with the C-terminus of Cnm67p in the two-hybrid system is Spc42, described by Adams and Kilmartin (1999). It was assumed that this interaction may not be direct and may also involve other SPB components. Both Cnm67p and Spc42p are associated in the Spc110p complex containing Spc110p, Spc42p, Spc29p, Cmd1p, and Cnm67p (Eliott *et al.*, 1999). Spc42p as well as Cnm67p are components of the cytoplasmic outer plaque of SPB but Spc42p forms a large central crystal and Cnm67p is present only in a small amount. It is supposed that Cnm67p is needed as a link between Spc42p and Spc29p/Nud1p. It is also postulated that before integration into SPB, Cnm67p is associated in the cytoplasm with another component – Spc94p, and it is this binary complex that is further integrated. The above conclusions were gained mostly from immunoprecipitation studies and some of

them were confirmed by two-hybrid analysis. In those studies the YOR129c protein was not reported. However, since our experiments indicate that this protein is present in the cell in a very small amount, it could not be easily detected during immunoprecipitation. When overexpressed, some components of SPB occupy whole or a significant part of the nucleus, e.g. Spc29p (Eliot *et al.*, 1999). It cannot exhibit growth defects when screened in standard conditions. However, when we carried out a broad phenotypic screen and compared the single *yor129c*Δ and *cnm67*Δ mutants with the *yor129c*Δ *cnm67*Δ double deletion strain we found some interactions. We found that the double deletion mutant exhibited moderately increased resistance to 0.1 M LiCl and 40 μg/ml hygromycin B compared with

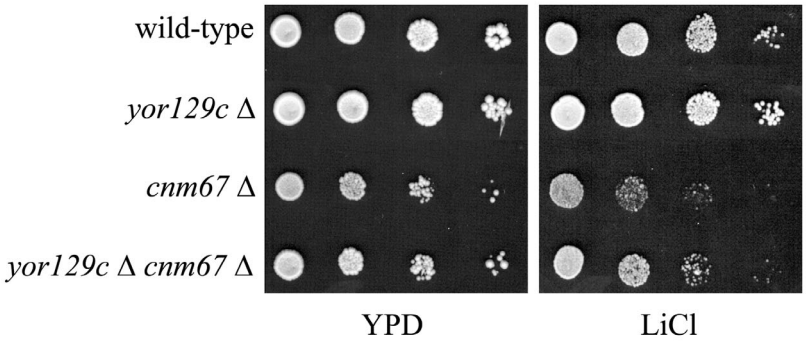

Figure 3. Deletion of *YOR129c* in the background of *cnm67*Δ strain increases lithium tolerance.

Cells were grown overnight in YPD, cultures were adjusted to 1×10^7 cells/ml and 10-fold serially diluted; 5 μl of each dilution was spotted on YPD and YPD + 0.1 M LiCl plates.

be excluded that the overexpression of *YOR129c* (from the *MET25* promoter) produced a similar picture, but at its native level the studied protein is associated with SPB only.

Genetic interactions between *CNM67* and *YOR129c*

Since our localization studies seemed to exclude the presence of the YOR129c protein uniquely in SPB, we reasoned that the results of the two-hybrid study could be artifactual. The possibility was strengthened by additional experiments, not described in this study, which showed that deletion of *YOR129c* does not influence cell division and the regularity of the microtubule cytoskeleton. Thus, to confirm the putative genetic interactions between these two proteins we constructed the double-deletion mutant *yor129c*Δ *cnm67*Δ. According to databases, the single deletion mutant *yor129c*Δ apparently does not the *cnm67*Δ single mutant. In Fig. 3 we present a drop-test result on YPD medium supplemented with 0.1 M LiCl. The result on medium supplemented with hygromycin B looked exactly the same (not shown). We also noticed that the *yor129c*Δ strain exhibited a decreased sensitivity to 0.1 M NaCl (not reported in databases), whereas in the double mutant this effect was lost and its growth on 0.1 M NaCl-supplemented medium was the same as that of the single mutant *cnm67*Δ.

Deletion of the *CNM67* gene causes spindle misorientation and impaired nuclear migration. As a consequence, bi- and multinucleated cells are observed. We checked that introducing the second deletion – *yor129c*Δ – did not revert this phenotype.

The single deletion of *YOR129c* does not cause any distinct phenotype. On the other hand, deletion of *CNM67* produces a whole spectrum of phenotypes accompanying the generally poor viability of the mutant severely disturbed in cell division. However, combin-

ing *yor129c*Δ and *cnm67*Δ improved the viability of the double mutant relative to the *cnm67*Δ strain. Moreover, compared to *cnm67*Δ, the double mutant exhibited slightly increased resistance to LiCl and to hygromycin B, but not to a whole spectrum of other agents tested.

In the yeast, homeostasis of Li^+ is maintained by multiple transport pathways also transporting Na^+, but the route of entry of both cations has not been defined yet. It has been proposed that both cations enter the cell through the K^+ transporter Trk1p (Gómez *et al.*, 1996). Increased sensitivity/resistance to lithium is usually accompanied by increased sensitivity/resistance to sodium (not observed in the *yor129c*Δ *cnm67*Δ strain). In a screen searching for genes conferring lithium tolerance upon overexpression the gene *SIT4* was identified (Masuda *et al.*, 2000). This gene was originally identified as a Ser/Thr protein phosphatase acting as a cell cycle regulator (Arndt *et al.*, 1989; Sutton *et al.*, 1991), but Masuda *et al.* (2000) demonstrated that it plays also a role in monovalent cation homeostasis. Its transcription is induced by lithium and this induction constitutes a regulatory mechanism of an as yet unknown function. It is known that *SIT4* regulates total cation content upon a Na^+ or Li^+ stress and this regulation is mediated *via* the K^+ efflux transporter (Masuda *et al.*, 2000). Recently a homologue of *SIT4* was isolated in the budding yeast *Kluyveromyces lactis*. In this organism *SIT4* has a broad role in regulating multidrug resistance (MDR) (Chen *et al.*, 2000) and it is supposed that the Sit4 protein phosphatase controls the activity of the ABC transporters specific for these drugs. It was shown that the MDR phenotype of *sit4* mutants of *K. lactis* is mediated *via* activation of *KlPDR5*, a homologue of *S. cerevisiae PDR5*, that is responsible for the efflux of oligomycin, antimycin, and the antifungal drugs ketoconazole and econazole (Chen, 2001).

The YOR129c protein described here shows some resemblance to Sit4p. It interacts with the Cnm67 protein participating significantly in cell divisions and it also influences salt and drug tolerance. Thus, it may be a good candidate for an accessory protein associated with the cytoplasmic face of SPB and playing a role in cation homeostasis and/or MDR.

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