**Dolichol phosphate mannose synthase from the pathogenic yeast Candida albicans is a multimeric enzyme.**

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**Abstract**

*Background*: Dolichol phosphate mannose synthase (DPMS) is a key enzyme in N- and O-linked glycosylations and glycosylphosphatidylinositol (GPI)-anchor synthesis. DPMS generates DPM, the substrate for mentioned processes, by the transfer of mannosyl residue from GDP-Man to dolichol phosphate. Here we describe the role of DPMS for Candida albicans physiology with emphasis on the cell wall composition and morphogenesis.
*Methods*: C. albicans genes for DPMS subunits were cloned, tagged and expressed in Saccharomyces cerevisiae. The C. albicans strains with controlled expression of DPM genes were constructed and analyzed. Gene expression and enzyme activities were measured using RT-PCR and radioactive substrate. Sensitivities against chemical agents
were tested with microdilution method. The composition of the cell wall was estimated by HPLC. Glycosylation status of the marker protein was analyzed by Western blot. Morphological differentiation of the strains was checked on the media promoting hyphae and chlamydospore formation.
*Results*: We demonstrate that C. albicans DPMS consists of three interacting subunits, among which Dpm1 and Dpm3 are indispensable, whereas Dpm2 increases enzymatic activity. Lowered expression of DPMS genes results in decreased DPMS activity, increased susceptibility to cell wall perturbing agents and in altered cell wall composition. Mutants Tetp-DPM1 and Tetp-DPM3 show defective protein glycosylation and are impaired in hyphae and chlamydospore formation.
*Major conclusion*: DPMS from C. albicans, opposite to S. cerevisiae, belongs to the family of DPMS with multimeric protein structure.
*General significance*: This work provides important data about factors required for a proper protein glycosylation and for morphogenesis of pathogenic yeast C. albicans.

**1. Introduction**Dolichol phosphate mannose (DPM) serves as a mannose donor for several enzymes acting in endoplasmic reticulum of eukaryotic cell. DPMS (EC 2.4.1.83), the enzyme catalyzing DPM formation by the transfer of mannose from GDP-Man to Dol-P is essential for yeast cell viability, while its mutation entails severe phenotype. DPMS activity is required for N-glycosylation, O-and C-mannosylation and GPI-anchor synthesis [1,2]. Moreover, the stimulating role of DPM on UDP-GlcNAc:Dol-P GlcNAc-1- phosphate transferase (GlcNacPT) involved in the first step of the dolichol lipid linked oligosaccharide (LLO) formation indicates regulatory relationship between DPM synthesis and N-glycosylation, where LLO serves as a substrate [3]. Reciprocally, GlcNAc-PP-Dol, a product of the first step of N-linked glycosylation, induces production of DPM and also inhibits its own biosynthesis. DPM is synthesized on the cytosolic surface of the ER membrane and then flipped onto the lumenal side. Intensively studied Saccharomyces cerevisiae dpm1-6 mutant, next to the expected glycosylation defects, is thermosensitive, susceptible to some chemicals and has disturbed cell wall structure and composition [1,4,5]. What is interesting, described phenotypes can be rescued by overexpression of RER2 gene, encoding cis-prenyltransferase Rer2 [5]. In human, the severe congenital disorders of glycosylation type 1e are caused by the defect in DPMS [6,7]. Due to the distinct structures, DPMS-es could be divided into two classes. The first class contains single-component enzymes with a hydrophobic stretch at the carboxyl-terminus which allows anchoring of the protein to the ER membrane. Such a structure was reported for DPMS from S. cerevisiae [4], Trypanosoma brucei [8], Ustilago maydis [9] or Leishmania mexicana [10]. The enzymes belonging to the second class are composed of three subunits. To this class belong human [11], Schizosaccharomyces pombe [12], Trichoderma reesei [13,14] or Arabidopsis thaliana DPMS [15]. The group of T. Kinoshita in a series of papers described functions and interactions of three subunits of human DPMS. They concluded that soluble catalytic Dpm1 subunit is stabilized and localized in the ER through binding with Dpm3, while Dpm3 protein is stabilized by binding with Dpm2 [11,16]. Therefore, Dpm3 is necessary for complex stability and in the absence of Dpm3, Dpm1 is degraded in a proteasome-dependent manner [17]. Dpm2 increases enzyme activity by enhanced binding of dolichol phosphate and probably regulates expression of Dpm1 [18]. Moreover, Dpm2 is also a dispensable component of the GPI-GnT (GPI-GlcNac transferase) enzyme complex, whose activity is enhanced in the presence of [19]. In this paper we present results indicating that the structure of Candida albicans DPMS is similar to human synthase i.e., it is a complex of tree subunits Dpm1p, Dpm2p and Dpm3p. We also describe the phenotypes deriving from a decreased expression of the particular DPMS subunits.

**Materials and methods**Yeast and bacterial strains used in this study
For plasmid propagation Escherichia coli strain DH5αF′ (F′ supE44
ΔlacU169 {ϕ80 lacZΔM15} hsdR17 recA1 endA1 gyrA96 thi-1 relA1)
[20] was used.

Yeast Saccharomyces cerevisiae strain

|  |  |  |
| --- | --- | --- |
| Y25598 | BY4743*; DPM1::kanMX4/DPM1* | Euroscarf |

Candida albicans strains

|  |  |  |
| --- | --- | --- |
| **Strain** | **Relevant genotype** | **Reference/Source** |
| CAF2-1 | *ura3Δ::imm434/URA3* | Fonzi & Irwin, 1993 |
| CAI4 | *ura3Δ::imm434/ ura3Δ::imm434* | Fonzi & Irwin, 1993 |
| DPM1/dpm1+URA | like CAI4 but *ORF19.5073/ orf19.5073::hisG-URA3-hisG* | this work |
| DPM1/dpm1-ura | like CAI4 but *ORF19.5073/ orf19.5073::hisG* | this work |
| TETp-DPM1 | like CAI4 but *TETp*-*ORF19.5073/ orf19.5073::hisG* | this work |
| DPM2/dpm2+URA | like CAI4 but *ORF19.1203.1/ orf19.1203.1::hisG-URA3-hisG* | this work |
| DPM2/dpm2-ura | like CAI4 but *ORF19.1203.1/ orf19.1203.1::hisG* | this work |
| TETp-DPM2 | like CAI4 but *TETp-ORF19.1203.1/ orf19.1203.1::hisG* | this work |
| DPM3/dpm3+URA | like CAI4 but *ORF19.4600.1/ orf19.4600.1::hisG-URA3-hisG* | this work |
| DPM3/dpm3-ura | like CAI4 but *ORF19.4600.1/ orf19.4600.1::hisG* | this work |
| TETp-DPM3 | like CAI4 but *TETp-ORF19.4600.1/ orf19.4600.1::hisG* | this work |

Media and growth conditions
E. coli was grown at 37 °C in solid or liquid LB medium (1% bactopeptone, 0.5% yeast extract, 1% NaCl) supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) when necessary. Yeast strains were routinely grown in a YPD medium (1% yeast extract, 1% peptone, 2% glucose) or SD medium (2% glucose, 0.67% yeast nitrogen base) supplemented with amino acids when required. To repress the tetracycline promoter different concentration of doxycycline (Sigma) was added to the medium. Solid media were prepared with 2% bacto-agar. Chlamydospore formation was tested on cornmeal agar with 0.5% Tween 80. Cultures were streaked on the agar, covered with cover glass and incubated at 25 °C for 7 days in darkness. FOA plates (2% glucose, 0.67% yeast nitrogen base, 0.1% 5-fluoroorotic acid, 10 μg/ml uridine, 2% agar) were used to force the excision of URA3 gene from C. albicans transformants. To sporulate, S. cerevisiae strains were cultivated on liquid medium containing 1% potassium acetate, 0.1% yeast extract
and 0.05% glucose for 7 days at 30 °C.
Primers
Primers used in this study are listed in Table S1.
Bioinformatics tools
The following tools were used for sequences analyses: BLAST implemented to Candida Genome Database (<http://www.candidagenome>. org/cgi-bin/compute/blast\_clade.pl) [22]; MAFFT (<http://mafft.cbrc.jp/> alignment/server/) [23]; JalView (http://www.jalview.org/) [24]; MatGAT (http://www.biomedcentral.com/content/supplementary/ 1471-2105-4-29-s1.zip) [25]; TMHMM (<http://www.cbs.dtu.dk/> services/TMHMM/) [26]; CCHMM (http://gpcr.biocomp.unibo.it/cgi/predictors/cc/pred\_cchmm.cgi) [27].
DNA and RNA manipulations
Preparations of bacterial plasmids were done by the alkaline lysis method. Restriction enzymes (Fermentas Standard and FastDigest®) and DNA modifying enzymes were used according to the suppliers' recommendations. For PCRs DreamTaq™ (Fermentas) or Phusion® High-fidelity DNA Polymerase (Finnzymes) were used. When necessary
plasmids and DNA fragments were purified with the Clean-up kit (A&A Biotechnology) or extracted from agarose gel with the Gel-out kit (A&A Biotechnology). DNA and RNA concentrations were measured on a NanoDrop Spectrophotometer (Thermo Scientific). Reverse transcription reactions were performed with the Advantage RT-for-PCR Kit (Clontech). All described genes and DNA fragments required for construction of deletion cassettes were inserted into pGEM-T Easy Vector (Promega) or Zero Blunt TOPO (Invitrogen) plasmid. DNA was sequenced by Laboratory of DNA Sequencing and Oligonucleotide Synthesis of Institute of Biochemistry and Biophysics, Warsaw.
C. albicans transformation was performed according to [28].
Plasmid and strain construction
All three genes encoding CaDPMS subunits were amplified by PCR from C. albicans cDNA with following primers pairs: HADPM1-F/ HADPM1-R, FLDPM2-F/FLDPM2-R and mycDPM3-F/mycDPM3-R. PCR products were subcloned to pCR-Blunt II-TOPO and sequenced. Subsequently, genes were ligated to pESC vectors in the following way:
CaDPM3 was cloned into XhoI/HindIII site of pESC-LEU in frame with c-myc tag. CaDPM2-containing fragment was cloned to BglII/SacI site of pESC-URA in frame with FLAG, and HA-tagged CaDPM1 (tag introduced in the primer) was ligated to the SalI/HindIII site of resulting plasmid.
In order to delete selected genes from C. albicans genome the URAblaster method was used [21]. In this method 5′ and 3′ regions of homology to gene of interest are amplified by PCR and cloned into the p5921 plasmid in this manner to flank hisG-URA3-hisG sequence and create a deletion cassette. The cassette is then cleaved from plasmid and used for transformation of ura-minus strain (CAI4). Prototrophic transformants are analyzed for correct cassette integration by PCR or Southern blot and spread on FOA plates to excise URA3 gene thanks to spontaneous recombination between the hisG repeats. Obtained uraminus segregants might be used in the second round of transformation. The 5′ flank and 3′ flank of orf19.5073 were amplified with following
primer pairs: DPM1F1F/DPM1F1R and DPM1F2F/DPM1F2R (respectively), then ligated into SacI/BglII and SalI/HindIII (respectively) sites of p5921. The SacI/HindIII fragment of the resulting plasmid served as a transformation cassette. To place the second copy of orf19.5073 under the control of tetracycline promoter primers DPM1TET-F and DPM1TET-R were used to amplify a cassette on a template of p2151c. The cassette contained homology fragments to target integration into orf19.5073, URA3 selective marker, the fusion transactivator – tetR- cHAP4AD placed under CaENO1 promoter and the regulatable tetOScHOP1 promoter. The cassette was used for transformation of DPM1/dpm1-ura strain.
In order to construct the cassette for orf19.1203.1 deletion, primer pairs DPM2F1F/DPM2F1R and DPM2F2F/DPM2F2R were used to amplify 5′ flank and 3′ flank of the gene, respectively. Fragments were ligated into SacI/BglII and BamHI/SalI sites (respectively) of p5921 plasmid, then SacI/SalI cassette was cleaved and used for transformation of CAI4 strain. The pair of primers DPM2TET-F and DPM2TET-R and plasmid p2151c were used to produce the cassette introducing tetracycline promoter upstream to the second allele of orf19.1203.1 in strain DPM2/dpm2-ura.
The 5′ and and 3′ flanks of orf19.4600.1 were amplified with the following primer pairs: DPM3F1F/DPM3F1R and DPM3F2F/DPM3F2R (respectively), then ligated into SacI/BglII and BamHI/SalI (respectively) sites of p5921. The SacI/SalI fragment of obtained plasmid served as a deletion cassette. In order to amplify the cassette for placing
orf19.4600.1 of strain DPM3/dpm3-ura under the control of TET promoter, primers DPM3TET-F and DPM3TET-R and plasmid p2151c as a template were used.
Cell wall preparation
Cells were harvested by centrifugation, washed with 10 mM Tris/HCl, pH 7.5, then suspended in the same buffer and fully disintegrated with 0.4–0.6 mm glass beads in the presence of a protease inhibitor mixture (Sigma). To remove non-covalently linked proteins and intracellular contaminants, isolated cell walls were washed extensively with ice-cold 1 M NaCl [29]. Subsequently cell walls were washed with miliQ water to remove salt and were lyophilized.
Cell wall chitin determination
For chitin measurements, alkaline hydrolysis of cell walls was performed in 6% KOH for 90 min at 80 °C in order to release cell wall proteins. After neutralization with acetic acid, cell walls were washed with phosphate buffered saline and chitinase buffer (18 mM citric
acid, 60 mM dibasic sodium phosphate, pH 6.0). Subsequently, the cells were treated with 0.33% chitinase C (InterSpex Products) for 3 h at 37 °C. The amount of N-acetylglucosamine liberated from chitin was measured with Ehrlich’s reagent as described [30].
High-performance anion-exchange chromatography analysis of cell wall
HPAEC analysis was performed using ICS-3000 Ion Chromatography System (Dionex). Cell wall was hydrolyzed with final concentration of 2 M TFA for 4 h at 100 °C with the presence of internal standard (fucose). After hydrolysis the mixture was evaporated under the stream of nitrogen then washed twice with methanol. The residue was dissolved in
deionized water and filtered on Amicon Ultrafree-MC Centrifuge Filter Device. Sample was applied on HPAEC CarboPac PA10 column, separated with 18 mM NaOH at a flow rate 0.25 ml/min and detection was accomplished with pulsed amperometric detection (PAD) [31].
Membrane fraction isolation
Collected cells were washed with 150 mM Tris–HCl pH 7.4 containing 15 mM MgCl2 and 9 mM β-mercaptoethanol, then suspended in the same buffer supplemented with proteinase inhibitor cocktail and vortexed vigorously with glass beads 8 × 1 min with intervals on ice. The homogenate was then centrifuged at 5000 ×g for 10 min to remove
debris and unbroken cells and supernatant was centrifuged for 1.5 h, 70 000 ×g at 4 °C. Obtained pellet was resuspended in 50 mM Tris/HCl pH 7.4, 3.5 mM MgCl2, 6 mM β-mercaptoethanol and homogenized in tissue grinder. Aliquoted membrane fraction was stored at −80 °C [32].
Determination of DPMS activity
Mannosyl transfer from GDP-mannose to Dol-P was measured in a 50 μl of reaction mixture containing 40 mM Tris/HCI, pH 7.4, 10 mM MgCl2, 100,000 c.p.m. GDP-[U-14C]mannose, 0.1% Nonidet NP-40, 5 μg dolichol phosphate (nonadecaprenol phosphate) and 100 μg membrane protein. The mixture was incubated at 30 °C for 5 min., then reaction was stopped by addition of 4 ml of chloroform/methanol (3:2, v/v),
washed once with 4 mM MgCl2 and washed twice with FUP solution (chloroform:methanol:water [3:48:47, v/v/v], 4 mM MgCl2]. The 1/10 part of the sample was evaporated and the radioactivity was measured by liquid scintillation counting [13,33].
Western blot analysis
Cell extract served as a source of protein and was obtained by vortexing yeast cell with glass beads and appropriate buffer. The homogenate was clarified by centrifugation (5 min, 15000 g) and stored at −80 °C for further analysis or mixed with a sample buffer (60 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 2% 2-mercaptoethanol, 0.0025%
Bromophenol Blue) and denaturated for 5 min at 100 °C. When membrane proteins were analyzed, the membrane fraction was used as a sample. Samples were loaded into the wells of the SDSpolyacrylamide gel (SDS-PAGE). Membrane proteins (300 μg) were
subjected to 10% SDS PAGE, transferred to the Immobilon P membrane (Milipore) and the Dpm proteins were detected by immunological reaction with a primary antibody. Depending on the secondary antibody used the results were visualized either by chromogenic substrates BCIP/NBT (when alkaline phosphatase coupled antibody was used) or by chemiluminescent substrate (Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate; for HRP-conjugated antibody used).
Co-immunoprecipitation
For co-immunoprecipitation the Dynabeads Protein A kit (Invitrogen) was used. Dynabeads Protein A were incubated in Ab binding and washing buffer for 15 min with approximately 4 μg of antibodies to allow the Dynabeads–Ab complex formation. Following a wash with Ab binding and washing buffer, the complex proceeded to immunoprecipitation. The membranes equivalent to 600 μg of proteins (estimated
by Lowry method) were solubilized in buffer A (0.5 M KCl, 1% OG [octyl b-D-glucopyranoside]) for 20 min on ice. Insolubilized remnants were pelleted (80000 ×g, 45 min, 4 °C), supernatant was added to Dynabeads–Ab complex and incubated with rotation for 1 h at room temperature to allow protein binding to antibody. Dynabeads–Ab–protein
complex was washed four times with buffer B (15 mM Tris–HCl, pH 7.5, 1.5 mM MgCl2, 150 mM NaCl, 5% glycerol, 0.5% OG) prior to elution of target proteins complex with 100 mM glycine pH 3.0. The eluate was neutralized by adding 1 M Tris–HCl pH 7.5, then run on SDS-PAGE gel and followed Western-blot procedures described above.

**Results**In silico analysis of C. albicans DPMS
Using sequences of S. pombe Dpms proteins as a query for BLAST tool available on Candida Genome Database (CGD) we identified C. albicans homologues of all three subunits: CaDpm1 encoded by ORF19.5073, CaDpm2 (ORF19.1203.1) and CaDpm3 (ORF19.4600.1). The comparison of amino-acid sequences revealed high similarity of C. albicans DPMS subunits to proteins from other species. Dpm1p expresses discernible
conservation even between such evolutionary distinct species as C. albicans and human (78.1% of similarity) (Fig. S1). Dpm1 is the catalytic subunit, thus the specific configuration of amino acids might be required for the enzyme activity. In accordance, among conserved amino acids of CaDpm1p we have found those recognized as important for substrate binding and catalysis in the yeast S. cerevisiae Dpm1p [34] (Fig. 1). It is noteworthy that prediction of transmembrane domains did not reveal any membrane-spanning helices in CaDpm1 protein. Thus, CaDpm1 is a soluble protein similar to Dpm1 subunits from other organisms possessing DPMS of a complex structure. When compared with other sequences available in CGD, we observed very high similarity (80%–100%) between C. albicans Dpm1p and homologous proteins from other Candida species with only one exception. Namely, Dpm1p from Candida glabrata is similar to CaDpm1 only in 47.5% and contains a transmembrane domain on its elongated carboxy-terminus and therefore might be classified to the “yeast” family of DPMS (Fig. 1). Accordingly, there were no homologues of Dpm2 and Dpm3 found for C. glabrata. In the mammalian cells Dpm1p is tethered to the ER membrane by Dpm3p, which possesses two transmembrane regions and coiled-coil domain at C-terminus, directly involved in the interaction between subunits [17]. Using bioinformatics tools (TMHMM and CCHMM) we found an analogous structure — two transmembrane domains (amino acids 7–29 and 39–61, see Fig. 1) and the coiled-coil domain (aa 63–82) for CaDpm3. CaDpm2, similarly known Dpm2 subunits from other organisms, contains two transmembrane regions (aa 7–29 and 49–71). The same conformation of Dpm2 and Dpm3 subunits was predicted for other species whose genomes are hosted on CGD (Fig. 1).
Thus our in silico analysis indicates that DPMS of C. albicans resemble the model described for mammalian class of the enzyme.
Experimental evidence for a complex structure of DPMS in C. albicans
The yeast S. cerevisiae serves as a common model for studying the gene function from other eukaryotes, thus we also used this organism to check functionality of Dpm1, Dpm2 and Dpm3 proteins, encoded by the respective genes from C. albicans.
To this end, we constructed plasmids bearing DPMS genes tagged with different epitopes at the N-terminus, and used them for further analyses. Obtained plasmids were used for transformation of Y25598 strain (dpm1::kanMX4/DPM1) and resulting transformants were sporulated. Tetrads analysis revealed that only two combinations (a) when all three subunits or (b) CaDPM1 with CaDPM3 were simultaneously expressed yielded four living spores (Fig. 2A). However, it should be noticed that not all tetrads gave four spores. Moreover, a higher number of viable tetrads were obtained when all subunits were expressed (50%) than in the case of the strain lacking CaDPM2 (19%). It indicates that
CaDPM2 helps spores to survive probably by improving functionality of DPMS. To test this idea, we selected S. cerevisiae spores expressing CaDpm proteins but lacking their native DPM1 gene. Subsequently, the in vitro activity of DPMS was measured in the membrane fraction of selected haploids. When compared with the spores bearing three subunits of DPMS, the spores lacking CaDpm2 show dramatically decreased enzymatic activity (by 75%; 9.1 ± 0.3 vs. 37.1 ± 9.6 [cpm ∗ min−1 ∗ μg protein− 1]), which may be responsible for lowered viability of the spores. Furthermore, the DPMS activity in the spores carrying C. albicans DPMS complex accounts for less than 20% of the activity
of the wild type S. cerevisiae spores (210.4 ± 34.5 cpm ∗ min−1 ∗ μg protein−1).
Summarizing, CaDpm1 and CaDpm3 proteins are necessary for the enzyme activity, while CaDpm2 is a modulating subunit not required for the cell viability.
It is noteworthy that spores expressing CaDpms proteins were supersensitive to Calcofluor White indicating severe changes in the cell wall integrity (Fig. 2). Thus, C. albicans DPMS complex can substitute yeast enzyme, however it does not fully restore the fitness of the cells. This might be due to the higher intrinsic activity of S. cerevisiae enzyme
or be a consequence of heterologous expression of tagged proteins.
Next, S. cerevisiae strains carrying three subunits of C. albicans DPMS complex were subjected to further analysis for possible interaction between the subunits using co-immunoprecipitation technique. To this end, the membrane fractions (source of proteins) from the strains Y25598 carrying plasmids pESC-URA [HA-CaDPM1, FLAG-CaDPM2]
and pESC-LEU [cmyc-CaDPM3] were solubilized using 1% octyl beta-D glucopyranoside to release the membrane-spanning subunits, Dpm2 and Dpm3. Then the proteins were immunoprecipitated with antiFLAG antibody–protein A–magnetic bead complex, targeted at FLAGCaDpm2, and washed to remove unbound and non-specific proteins. The precipitate was subjected to Western analysis utilizing antibodies
against specific tags i.e., anti-HA (Dpm1), anti-FLAG (Dpm2) and antic-myc (Dpm3). In the precipitate all three subunits were detected and demonstrate that they physically interact with each other (Fig. 3).
Functional analysis of the C. albicans genes encoding DPMS complex
To study the function of CaDPMS in the native organism we constructed mutated strains of C. albicans, each having a particular subunit of DPMS under the control of tetracycline regulated promoter. At first, one chromosomal copy of the selected gene was deleted with the “ura-blaster” cassette. Next, the URA3 marker was excised by cultivating cells on the plates containing 5-FOA, followed by transformation with a PCR product carrying TET promoter resulting in the tetracycline promoter integration to the specified locus (for more detail see Materials and methods section). The correct strain construction was confirmed
by Southern blot (not shown). In the obtained strains TETp-DPM1, TETp-DPM2 and TETp-DPM3 the expression of the respective gene should be repressed in the presence of doxycycline. To confirm this, the strains were cultivated to OD600 = 1.0–1.5 in YPD medium without or with addition of 10 μg/ml doxycycline. RNA from collected cells was
extracted and transcribed to cDNA. The resulting cDNA served as a template for PCR reactions targeted at ACT1 (housekeeping gene) and respective DPM genes. It was found, as expected, that the expression of DPM genes in constructed strains was tightly regulated by tetracycline promoter, since virtually no RT-PCR product of DPM genes was detected when cells were grown in the presence of doxycycline (Fig. S2).
Subsequently, the growth of the mutants was tested on YPD agar. In non-repressive conditions all strains grew similarly. When 10 μg/ml of doxycycline was added to the medium only the wild type strain grew identically with that in non-repressive conditions. TETp-DPM1 and TETp-DPM3 strains were not able to grow at all, while the growth of
TETp-DPM2 was slightly retarded (Fig. 4). Additionally, the growth defect of TETp-DPM1 and TETp-DPM3 was gradually diminishing with decreasing doxycycline concentration (not shown). The corresponding results were obtained for cultures in liquid YPD medium (not shown).
Next, the in vitro activity of DPMS was measured in the membrane fraction from TETp-DPM1-3 and from the wild type (CAF2-1) strains, cultivated overnight at 28 °C in non-repressive or repressive conditions. DPMS activity of CAF2-1 was not affected by the presence of doxycycline. The DPMS enzymatic activity of conditional mutants grown in
non-repressive condition was lowered by approximately 35% when compared with the wild type. However, in the presence of doxycycline the DPMS activity in TETp-DPM1 and TETp-DPM3 was barely detected, while in TETp-DPM2 it was decreased to 30% of the wild-type level (Fig. 4). This is consistent with the observed growth defect of strains
and matches also results we obtained in S. cerevisiae, where the absence of CaDpm1p or CaDpm3p results in the cell death but lack of CaDPM2 reduces the cell fitness and lowers DPMS activity (compare Fig. 2).
Considering the role of DPM for protein O- and N-glycosylation we checked the glycosylation status of the marker protein in TETp-DPM mutants. For this purpose we performed the Western blot analysis using anti-Gas1p antibodies to detect C. albicans Phr proteins (orthologs of ScGas1) in strains grown in repressive and non-repressive conditions. Since the final pH of the culture was below 5, we assumed that observed
form was Phr2, which was reported to be expressed in acidic conditions [35,36]. All tested strains grown in YPD without doxycycline gave similar results i.e., the strong band was visible at the position corresponding to approx. 110 kDa (Fig. 5). In the repressive conditions, however, the 110 kDa band was absent for TETp-DPM1 and TETpDPM3 strains while a slighter signal at 70 kDa, corresponding to underglycosylated protein, appeared. However, the glycosylation pattern of the Phr protein in TETp-DPM2 strain in the presence of doxycycline indicates that the diminished DPMS activity results in the formation of the additional glycoform (approx. 100 kDa).
The cell wall composition depends on DPMS activity
In C. albicans the mannoproteins constitute 35–40% cell wall dry weight and form outer, electron dense layer of the cell wall [37]. Mannoproteins are produced in N- and O-glycosylation pathways where DPM plays the main role as a mannose donor. Thus diminished activity of DPMS should lead to the decreased amount of mannose in the cell wall. To evaluate the cell wall composition of TET-DPM and wild type strains we used high-performance anion-exchange chromatography (HPAEC). Cell wall isolated from the cells cultivated in the presence or absence of doxycycline were hydrolyzed with
trifluoroacetic acid and applied on a column along with fucose as an internal standard. Since it was published that TFA liberates only a small part of N-acetylglucosamine from chitin chains [38], the amount of N-acetylglucosamine released from the cell wall by chitinase C was additionally determined using an Ehrlich reagent.
The HPAEC analysis revealed that switching off the transcription of genes encoding DPMS subunits results in significant changes in the composition of the cell wall. Interestingly, even in non-repressive conditions the amount of mannose in the cell wall of mutants was decreased by up to 23% when compared with the wild type strain. What is important is when cells were cultivated with doxycycline the amount of mannose in the cell wall of mutants dropped further reaching less than a half of the control level. The presence of doxycycline did not alter the composition of the CAF2-1 cell wall (Fig. 6A).
Mannan fraction is often decorated with the phosphate residues determining the negative charge of the cell wall. The phosphomannan fraction consists of up to fourteen β-1,2-linked mannose residues attached to the side chains of N-glycan or to O-linked mannan [39,40]. To test if the aforementioned component of the cell wall is affected by a decreased expression of DPMS, the capability of binding the positively charged dye Alcian Blue was checked. Interestingly, all conditional mutants were far less competent to bind Alcian Blue (in comparison to the wild type) similarly in both repressive and non-repressive conditions (Fig. 6B). These results suggest that fully active DPMS is required
for normal phosphomannan fraction synthesis and distribution.
As presented in the Fig. 6C, the amount of chitin in the wild type strain was constant regardless of the doxycycline addition. In the cell wall of TETp-DPM1 and TETp-DPM3 conditional mutants cultivated without doxycycline, the chitin level was elevated by app. 50% when compared with the wild type control, while the chitin level in the TETp-DPM2 cell wall was similar to the CAF2-1. Dramatic changes occur in the cell wall of all DPMS mutants grown in repressive condition — the chitin amount raised to 300% of wild type strain level. High chitin level was reported to be a consequence of defects in the synthesis of other components of the C. albicans cell wall i.e., glucans [41,42] and mannoproteins [43].

Sensitivity of C. albicans DPMS mutants to external agents
As presented above, C. albicans DPMS mutants exhibit alternated cell wall composition. We also observed the correlation of the latter with the susceptibility of the mutants to the various external agents (Fig. 7). At first, the concentration of doxycycline which slightly attenuates the growth of TETp-DPM1 and TETp-DPM3 was estimated at 30 ng/ml. Then series of two-fold dilution of stressing agents in YPD were prepared in 96-well sterile plates followed by inoculation with cell suspension. Plates were incubated at 28 °C for 20h, and the optical density at 600 nm was red on a microplate reader. When strains were
grown in the presence of cell wall perturbing agent Congo Red (CR), the growth of DPMS conditional mutants was affected even when doxycycline was not added. The susceptibility of mutants was enhanced in repressive conditions, where differences in the cell density between the wild type and TETp-DPM1 or TETp-DPM3 were observed at the concentration for as low as 6 μg of CR per ml. The sensitivity of TETp-DPM2 was visible at higher concentrations of CR, while the wild type was not affected over the entire tested CR concentration range.

Another compound, Calcofluor White (CFW) is known to bind chitin micelles and inhibit cell wall synthesis. We observed only slight differences in growth rate between strains cultivated in non-repressive conditions. On the contrary, when doxycycline was present in the medium, the divergence in growth curves between CAF2-1 and DPMS mutants was
evident with the highest difference at CFW concentration of 25 μg/ml. The susceptibility of TETp-DPM2 strain was slightly lower than two other mutants.
The growth of conditional mutants in the presence of hygromycin B was faintly retarded (compared with the wild-type strain) when nonrepressive conditions were applied. When cells were cultivated with doxycycline, the enhanced influence of hygromycin B on the growth of DPMS mutants, but not on the wild type, was observed (Fig. 7). Again, the susceptibility of TETp-DPM2 strain was not as striking as TETp-DPM1 or TETp-DPM3 mutants, which directly reflects the significance of particular DPMS subunits for enzyme activity.
Defect in DPM formation alters morphology of C. albicans
The morphological variation exhibited by C. albicans is a subject of multiple research. Since it is postulated that different forms have distinct properties allowing survival and propagation in various niches of the host organism, it is highly desirable to get knowledge about mechanisms leading to morphological switching.
As presented in Fig. 8, a horse serum stimulated the hyphal growth of all tested strains but in an unequal manner. The most extensive hyphae were formed by wild type CAF2-1. TETp-DPM2 strain produced a bit less abundant but still prominent hyphae, while TETp-DPM1 and TETp-DPM3 gave only poor filaments. In contrast, in the presence of
doxycycline (30 ng/ml) all mutants were not able to produce hyphae at all, while the wild type filamented identically as in the control conditions. It should be pointed out that the failure in filamentation does not result from the growth defect, because the size of arose colonies was comparable in both repressive and non-repressive conditions.
Another medium stimulating filamentation of C. albicans, Spider medium, contains mannitol as a sole carbon source. The wild type CAF2-1 showed abundant filamentous growth after five days at 37 °C on either doxycycline supplemented or not supplemented Spider plates. On the contrary, none of the conditional mutants formed filaments irrespective of doxycycline presence (Fig. 8).
We tested also the influence of DPM synthesis deficiency on chlamydospore formation. After 7 days of cultivation in 25 °C in darkness on cornmeal agar supplemented with 0.5% Tween 80 all strains developed filaments with chlamydospores at their tips and lateral branches (Fig. 8). When they grew in the presence of 30 ng/ml doxycycline, only wild type and TETp-DPM2 formed chlamydospores. TETp-DPM1 and TETp-DPM3 mutants developed only few short filaments which did not carry chlamydospores.
**Discussion**Mannose is the most abundant sugar component of yeast glycoproteins. However, GDPMan, a widely used mannosyl donor, is absent in the lumen of endoplasmic reticulum although in this compartment mannose residues are transferred to the core structures of N-glycans and GPI-anchor or directly to the protein in O-glycosylation process. The immediate mannosyl- donor for these reactions is DPM synthesized from Dol-P and GDP-Man on the cytoplasmic side of ER by essential enzyme DPMS. This enzyme could be a single component (e.g., yeast Dpm1) or consists of three subunits (e.g., human DPM1, DPM2 and DPM3) proteins, but the reason of existence of two DPMS classes is unknown. Based on a homology with enzymes from other organisms, we found that C. albicans DPMS is a complex of three subunits. Moreover, we analyzed the importance of DPM proteins for C. albicans morphogenesis, cell wall composition and protein glycosylation by constructing mutants with tetracycline-regulatable expression of particular DPMS
subunits.
In silico analysis shows that the DPMS of C. albicans resembles the model described for the mammalian class of enzyme [11].
In the present work, by co-immunoprecipitation procedure, we demonstrated that CaDPMS subunits interacts with each other (Fig. 3). Moreover, we prove that the expression of CaDPM1 alone was not sufficient to rescue the lethal deletion of S. cerevisiae DPM1 (Fig. 2), similar to human and S. pombe DPM1 [12] or T. reseei dpm1 [14]. When expressed in S. cerevisiae CaDPM1 together with CaDPM3 were essential for the formation of functional DPMS complex which suppressed ScΔdpm1 phenotype, whereas additional presence of CaDpm2 increased the enzymatic activity and improved cell fitness. In agreement, the growth of C. albicans strains with tetracycline regulatable (TET-OFF) expression of CaDPM1 or CaDPM3 was severely affected in the conditions
repressing the TET promoter (in the presence of doxycycline) and the DPMS activity was almost completely abolished, while shutting off the expression of CaDPM2 resulted in a moderate growth defect and in the reduction of DPMS activity. The positive effect of subunit 2 of DPMS on activity also showed on human [11] and A. thaliana enzymes
[15]. On the other hand, Zembek et al. [14] reported opposite properties of T. reseei DPMII, since the presence of Dpm2 subunit decreased the enzymatic activity of TrDPMS expressed in S. cerevisiae. Surprisingly, a combination of DPM1 and DPM2 proteins from A. thaliana is functional, but the activity is lowered by 95% when compared with the combination of all three subunits [15]. Thus, although the general structure of DPMS complex is similar between species, the influence of subunits 2 and 3 on enzyme properties differs among organisms. These facts incline to the idea that DPMS evolved from a single protein to the multicomponent system which is more applicable for regulation. We have additionally found that close taxonomic relationship between
C. glabrata and S. cerevisiae [44] concerns also DPMS structure, since both species possess a single-component enzyme of high similarity.
S. cerevisiae dpm1-6 mutant displays changes in the cell wall composition and structure [5]. The increase in chitin content was abrogated by overexpression of the wild copy of DPM1 or cis-prenyltransferase encoded by RER2 gene, clearly demonstrating the relationship between glycosylation and the cell wall construction. C. albicans conditional
mutants in DPMS described in the present paper also possess high level of chitin when cultivated in repressive conditions, however the scale of changes is much more prominent than in S. cerevisiae mutant (~3-fold versus 1.6-fold increase). Interestingly, TETp-DPM1 and TETp-DPM3 strains show the chitin amount elevated in comparison
with wild type even when grown in non-repressive conditions, manifesting the importance of subunits 1 and 3 for DPMS functionality. The changed cell wall composition is common for glycosylation mutants and is usually related to the increased sensitivity to Calcofluor White or Congo Red, agents that bind cell wall components [40,43,45]. Accordingly, strains with repressed expression of DPMS subunits were more susceptible to CFW and CR than the wild type; however, TETp-DPM2 strain was more resistant than two other mutants, pinpointing redundancy of the regulatory subunit. Interestingly, such a tendency was not observed when mannose content was considered. All three DPMS mutants grown in repressive conditions presented similar mannose:glucose ratio (app. 1:3.8) which was significantly lower than in the wild type strain (app. 1:1.1) (Fig. 6). It shows that the DPMS activity remaining after the DPM2 repression (~30% of the wild type) is not sufficient to correct a general cell wall mannosylation defect. However, the glycosylation status checked specifically for Phr2 protein indicates differences between
the strains, i.e., the presence of underglycosylated form for TETp-DPM1 and TETp-DPM3 but not for TETp-DPM2 strain. These intriguing results suggest the existence of preferences in the distribution of mannose residues in the case of Dol-P-Man deficiency.
The aforementioned glycosylation defect revealed by the immunoblot analysis concerns both quality and quantity of Phr2p. In TETp-DPM1 and TETp-DPM3 mutants cultivated with doxycycline we observed only a small amount of the marker protein with lower
molecular weight in comparison with the wild type or nonrepressive conditions. Therefore, a shortage in DPM level must result in defects in N-, O-glycosylation and GPI anchor synthesis which leads to instability of underglycosylated Phr2p like in MET3p-RER2 mutant [46].
Further, our results demonstrate that filamentous growth and chlamydospore production on different media have distinct requirements of DPMS gene expression and/or DPMS activity and related glycosylation processes. For instance, in non-repressive conditions filamentation on serum plates is less abundant in TETp-DPM1 and TETp-DPM3 than in
TETp-DPM2, while no differences between strains were observed in chlamydospore production. Additionally, TETp-DPM2 strain in the presence of doxycycline can produce chlamydospores but not hyphae. It can be assumed that hyphae formation demands higher DPM availability than chlamydospore production. This is supported by the fact that mannose is almost completely absent in chlamydospores [47], while many hyphae specific proteins are highly mannosylated cell wall proteins [48,49]. Moreover, completely abolished hyphae formation on Spider medium (but only partially reduced on serum plates) reveals haploinsufficient phenotype of DPMS mutants and indicates
different mechanisms of the filamentation induction driven by serum and mannitol.
Summarizing, we demonstrated that DPMS in C. albicans is a complex of three Dpm subunits. Dpm2 subunit is not essential, however, its absence resulted in the significant drop of DPMS activity.
Moreover, due to its distinct role in protein glycosylation and cell wall integrity DPMS activity is crucial for C. albicans morphological differentiation.

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**Figure legend**

Fig. 1.

Comparison of amino acid sequences of dolichol phosphate mannose synthase subunits from indicated species. The sequences of S. cerevisiae (Dpm1, NP\_015509.1) and S. pombe (DPM1, NP\_594017.1; DPM2, NP\_595676.1; DPM3, NP\_596640.1) were obtained from NCBI database. The rest of the sequences were identified using CGD built-in BLAST tool with S. pombe sequences as a query. MAFFT tool was used to compare sequences. The JalView program was used to color graphs (darker indicates higher conservation). The highly conserved amino acids of subunit 1 important for activity are marked with a triangle; amino acids forming predicted transmembrane domains are underlined; predicted coiled-coil domains within subunit 3 are placed in boxes.

Fig. 2.

Dolichol phosphate mannose synthase complex from C. albicans suppresses the lethal deletion of DPM1 gene in S. cerevisiae. A. The Y25598 strain (DPM1::kanMX4/DPM1) was transformed with plasmids bearing indicated genes and sporulated and tetrads were dissected on YPGal plates. B. The spores of Y25598 strain expressing either native ScDPM1 gene or indicated combinations of C. albicans DPM genes were grown overnight in YPGal, then series of tenfold dilution were spotted on YPGal supplemented or not with 10 μg/ml of Calcofluor White.

Fig. 3.

Physical interaction of CaDPMS subunits. The solubilized membrane fraction of the
haploid spore (ScDPM1::kanMX4) bearing tagged CaDpm proteins served as a protein
source for co-immunoprecipitation experiment. Proteins were immunoprecipitated with
anti-FLAG antibody and subjected to anti-cmyc, anti-HA and anti-FLAG Western blot
analysis along with solubilized membranes (input) and wash fractions.

Fig. 4.

The growth (left) and Dol-P-Man synthase activity of TETp-DPM strains (right). Ten-fold dilutions of indicated strains were applied on YPD or YPD + 10 μg/ml doxycycline and
incubated at 28 °C for 48 h. DPMS activity was measured in the membrane fraction of indicated strains grown in liquid YPD with or without doxycycline. Activity of the wild type strain cultivated in YPD is used as a reference (100% of activity).

Fig. 5.

Anti-Gas1 Western analysis reveals protein glycosylation defects of C. albicans Dol-P-Man synthase mutants. Indicated strains were grown in YPD or YPD + doxycycline at 28 °C. The cells were broken with glass beads and cell-free extract was resolved on 8% SDS-PAGE gel. After electrotransfer of proteins, the PVDF membrane was subjected to immunodetection with anti-Gas1p rabbit antibodies.

Fig. 6.

Low Dol-P-Man synthase activity alters composition of the C. albicans cell wall.

A. The amount of major carbohydrates was measured with High Performance AnionExchange Chromatography in the cell wall digested with 2 M TFA. The means of results from two independent experiments are presented.

B. Alcian Blue binding by conditional mutants in Dol-P-Man synthase. Two OD units of cells of indicated strains were incubated with Alcian Blue solutions, pelleted and washed. The percentage of initial Alcian Blue precipitated with cells is presented on the graph.

C. The amount of chitin in the cell wall. The cell wall of indicated strains was treated with 0.33% chitinase C and liberated N-acetylglucosamine was measured by the colorimetric method as described in the Materials and methods section. Cells used for cell wall isolation were grown in YPD supplemented or not with 10 μg/ml doxycycline.

Fig. 7.

Sensitivity of Dol-P-Man conditional mutants to selected compounds. Two fold dilutions of indicated chemicals in YPD medium were prepared across the multi-well plates. Tested
strains were suspended in YPD or YPD + doxycycline and used for inoculation. After 24 h the optical density was measured. The OD 600 of a particular strain grown in the absence of a tested agent is presented as a 100%.

Fig. 8.

A morphological differentiation of C. albicans depends on DPMS activity. Strains were streaked on indicated media and colonies were photographed after 3 days at 37 °C (YPSerum), 5 days at 37 °C (spider) or 7 days at 25 °C (cornmeal agar). The following magnification was used to take pictures: 5× (YPSerum), 10× (spider) and 40× (cornmeal agar).