Research Article

FUNCTIONAL CHARACTERIZATION OF SPERMINE FAMILY TRANSPORTER *caf5*⁺ IN *Schizosaccharomyces pombe* (Lindner)

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Abstract: Polyamines are well conserved polycationic molecules that are known to interact with nucleic acids and contribute to multiple functions including cell cycle and stress response. The transport of polyamines in and out of the cell is driven by polyamine transporters that play a significant role in polyamine homeostasis. *Schizosaccharomyces pombe* (Lindner) *caf5*⁺ gene codes for a spermine family transporter that is yet to be characterized functionally. This study aims to understand the contribution of *caf5*⁺ on different processes previously associated with polyamines, by reverse genetics. Deletion mutants of *caf5*⁺, which are viable in normal conditions, were scanned for multiple cellular processes. The results showed that *caf5*⁺ deletion caused shorter cell length and slightly faster growth rate at the optimum conditions. *caf5Δ* cells also showed sensitivity to high doses of UV irradiation, while no sensitivity was observed against osmotic stress or another DNA damaging agent hydroxyurea. The mutants could successfully go through different phases of mitosis and meiosis as observed by DNA and septum staining. In summary, *caf5*⁺ gene is involved in normal growth and cell cycle progression, as well as stress response upon UV irradiation.

Key words: Schizosaccharomyces pombe, cell size, cell cycle, stress response, polyamine.

Özet: Poliaminler, nükleik asitlerle etkileştiği ve hücre döngüsü ile stres tepkisi gibi pek çok hücresel işleve katıldığı bilinen, korunmuş polikatyonik moleküllerdir. Poliaminlerin hücre içine girişi ve hücre dışına çıkışı, poliamin homeostazında görevli olduğu bilinen poliamin taşıyıcı proteinleri tarafından yürütülmektedir. *Schizosaccharomyces pombe* (Lindner) *caf5*⁺ geni de henüz işlevsel olarak karakterize edilmemiş bir spermin ailesi taşıyıcısıdır. Bu çalışmanın amacı ters genetik yöntemlerle *caf5*⁺ geninin poliaminlerle ilişkili olduğu bilinen hücresel işlevler üzerindeki önemini anlamaktır. Normal koşullarda yaşayabilir durumda olan, *caf5*⁺ geni delesyon mutantları (*caf5Δ*) pek çok farklı hücresel işlev üzerinden taranmışlardır. Sonuçlar, *caf5*⁺ geni delesyon mutantlarının hücre boyunun daha kısa olduğunu ve optimum koşullar altında daha hızlı bölündüklerini göstermektedir. *caf5Δ* hücreler, aynı zamanda yüksek dozda UV ışınlarına karşı hassasiyet göstermişler ancak ozmotik stres koşullarında ve bir başka DNA hasarı ajanı olan hidroksiüre'ye karşı herhangi bir hassasiyet göstermemişlerdir. DNA ve septum boyamaları sonucunda bu mutantların mitoz ve mayoz bölünmenin farklı fazlarını başarılı bir şekilde tamamlayabilir olduğu bulunmuştur. Özetle, *caf5*⁺ geni normal hücre büyümesinde, hücre döngüsünde ve UV ile indüklenen stres tepkisinde rol oynamaktadır.

Introduction

Polyamines are small, ubiquitious polycations that can be found in every organism except Archae, Methanobacteriales and Halobacteriales (Hamana & Matsuzaki 1992). Spermine and spermidine are the most common polyamines, but there are also polyamines in thermophilic organisms in the form of long or branched hydrocarbon chains (Fukuda *et al*. 2015).

Polyamines are involved in multiple functions in the cells such as stabilizing nucleic acids, regulation of gene expression, cell cycle, stress response and pathogenic activity. Polycationic nature of polyamines enables their binding to anionic molecules in the cells and most of the intracellular polyamines are found as polyamine-RNA complexes. The ability of polyamines to readily bind to DNA and RNA contributes to nucleic acid stability (Katz *et al.* 2017). Stabilization of DNA by polyamines is especially important for heat resistance, and hence survival of thermophilic microorganisms at extreme temperatures (Fukuda *et al.* 2015). Polyamines also contribute to the regulation of gene expression at the level of translation by different mechanisms including formation of the initiation complex, facilitating fMet-tRNA binding in bacteria or by supporting post translational modification of the translation factor eIF5A in yeasts (Gevrekci 2017). Among others, the role of polyamines in providing resistance to environmental stress is best characterized in a number of organisms including bacteria, yeasts, fungi and plants. Polyamine

mutants were shown to be sensitive to oxidative stress in *Ustilago maydis* (DC.) Corda and *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Balasundaram 1993, Valdés-Santiago 2010), while putrescine level was shown to increase upon oxidative stress in *Escherichia coli* T. Escherich (Ucisik-Akkaya *et al.* 2014). Similar results were reported also for osmotic stress, showing that *U. maydis* polyamine synthase gene mutants (Valdés-Santiago 2009) and yeast polyamine uptake regulator gene (*pts2)* mutants were sensitive to osmotic stress (Erez & Kahana 2002). Moreover, *S. cerevisiae* and *Synechocystis* sp. were shown to respond to osmotic stress by regulating polyamine transport, showing the role of polyamines in osmotic stress response (Aouida *et al.* 2005, Jantaro *et al.* 2003, Lee *et al.* 2002).

Another significant role assigned to polyamines is the regulation of cell cycle progression. Cell cycle is a unidirectional series of events that initiates with monitoring the intracellular and extracellular environment and finally leads to the formation of two identical copies of the cell. The involvement of polyamines in cell cycle control is mostly characterized in higher eukaryotes although there are several examples among microorganisms. Inhibition of polyamine biosynthesis prolonged the S phase in the Chinese hamster (*Cricetulus griseus* Milne-Edwards) ovary (Fredlund & Oredsson 1996) and arrested the cell cycle at G1 phase in intestinal epithelial cells (Ray *et al.* 1996). Additionally, polyamine depletion caused T lymphoblastic cells to arrest at the G1 phase (Choi *et al.* 2000). The significance of polyamines in cell cycle regulation was also shown in *Schizosaccharomyces pombe* (Lindner). Depletion of polyamines at the early phases of the cell cycle was shown to induce G1 arrest. Upon prolonged polyamine deprivation, however, *S. pombe* cells showed a number of cell cycle phenotypes such as disruption of the actin network, disintegration of the nucleus and absence of septum (Chattopadhyay *et al.* 2002).

Since polyamines are involved in multiple different functions in the cells, polyamine homeostasis should be well established. A number of mechanisms have evolved to keep the intracellular polyamine levels at optimum levels. Polyamine biosynthesis (*de novo* or by interconversion of polyamine molecules) and transport are the two major ways to supply the right amount of polyamine to the cells. The most common biosynthetic pathway initiates with conversion of L-ornithine into putrescine by ornithine decarboxylase (ODC), followed by spermidine and spermine formation by the consequitive activities of spermidine synthase (SpdSyn) and spermine synthase (SpmSyn) (Michael 2016). In addition to the biosynthetic pathway, a number of polyamine family transporters also contribute to the regulation of intracellular polyamine concentration. Spermine and spermidine family transporters are transmembrane proteins that can be found in organelle or plasma membranes, driving polyamine influx or efflux. The efflux of polyamines was shown to be induced with decreased growth rate and inhibited upon increased growth rate (Wallace & Keir 1981, Wallace & Mackarel 1998). This fact signifies the role of polyamine transporters in the cells along with the study that showed yeast polyamine transporter Tpo1 extends the cell cycle arrest induced upon environmental osmotic stress (Krüger *et al.* 2013).

The present study was performed in order to identify and functionally characterize spermine family transporter *caf5⁺* in *S. pombe*. For this purpose, the deletion mutant of *caf5⁺* gene was used. The mutant was scanned for normal growth rate, stress response, cell division defects and spore formation to determine any process that was defective in the absence of this gene, as an indicator of its involvement in that particular process.

Materials and Methods

Schizosaccharomyces pombe Strains and the Media

Schizosaccharomyces pombe 972 *(h⁻ ade)* strain was used to form *caf5⁺* deletion mutants and as wild-type control. The strains were handled as explained in Moreno *et al.* (1991). The wild type and mutant strains were incubated in yeast extract agar (YEA) plain media (5 g/l difco yeast extract, 30 g/l glucose, 75 mg/l adenine, pH adjusted to 5.6 with HCl – supplemented with 2% (w/v) agar for agar media). The YEA agar was supplied with 1 M KCl, 120 mM CaCl₂ or 2 M Sorbitol for the osmotic stress response experiment. For hydroxyurea treatment, the media was supplied with 4 M hydroxyurea. A series of different concentrations of the stress agents (both salts and hydroxyurea) was initially used for stress response experiments. Specifically, the concentrations started with 0.5 M KCl, 60 mM CaCl₂, 1 M Sorbitol and 2 mM hydroxyurea and systematically increased up till 2M KCl, 240 mM CaCl₂, 4 M Sorbitol and 10 mM hydroxyurea in which even the wild type cells couldn't grow properly (so not informative to detect any difference in growth rate). Thus, the maximum salt and hydroxyurea concentrations that allowed proper wild type cell growth were shown in this study. To induce sporulation, the cells were streaked onto sporulation agar (SPA) medium [10 g/l glucose, 1 g/l KH₂PO₄, 1 ml/l 1000 X vitamin stock (1 g/l panthothenate, 10 g/l nicotinic acid, 10 g/l inositol, 10 mg/l biotin)]. The SPA medium causes nitrogen starvation, which consequently induces G1 arrest, mating (in the presence of the opposite mating type) and finally meiosis to form spores.

Forming Single Gene Mutations in S. pombe

caf5⁺ gene deletion is performed by a PCR-based method explained in Bähler *et al.* (1998). pFA6a-kanMX6 gene deletion cassette containing kanamycin resistance gene was amplified by specific primers with complementary sequences to the immediate upstream and downstream regions of *caf5⁺* gene. Kanamycin resistance gene provides resistance to G418 in *S. pombe*. Upon transformation of the deletion cassette by LioAc method of transformation, the cells were initially plated onto YEA agar and after 24 hours at 30°C, they were replica-plated

onto YEA plate supplemented with 200 mg/l G418 to select for the kanamycin resistance cassette. The colonies which were resistant to G418 showed up in 3-5 days incubation at 30°C, which were then tested by colony PCR for the genomic location of the deletion cassette.

Colony PCR

The cells surviving in the G418 containing YEA agar are known to have deletion cassette in the genome. To make sure that the deletion cassette was inserted at the right location in the genome, hence the right portion of the genome was deleted, colony PCR was used. The procedure started with boiling the colonies with dNTP mix, PCR buffer and the primers, at 98°C for 10 min. After being cooled down on ice, Taq polymerase was added and the following PCR program was run: 30 cycles of 94°C for 20 s, 50°C for 40 s, and 72°C for 1 min/kb, followed by 72°C for 5 min. The expected size of the product was 305 kb for *caf5⁺* deletion. The forward primer was designed according to the upstream region of *caf5⁺* gene and the reverse primer was designed according to the deletion cassette.

Forming Double Mutants in S. pombe

caf5Δ SPBC409.05Δ double mutants were formed by crossing *h⁻ caf5Δ* and *h⁺ SPBC409.05Δ* cells, which were resistant to G418 and hygromycin, respectively. The single mutants were initially streaked together on SPA agar sporulation media and incubated for 3 days for G1 arrest, mating and spore formation. The cells were then replica-plated onto YEA + 200 mg/l G418 and incubated at 30°C for 3 days. Then the colonies were replica-plated onto YEA + 300 μg/ml hygromycin. The colonies that could grow in both of the agar plates were expected to have both of the deletion cassettes, and hence neither of the *caf5⁺* and *SPBC409.05* genes.

Growth Rate and Cell Size Analysis

For the growth rate analysis, the cells were inoculated into YEA broth and incubated overnight (O/N) at 30°C. When they reached the fast growing phase, the cells were diluted to 10^6 cells/ml and t = 0 timepoint samples were collected. Additional samples were collected at t = 2 hr, t = 4 hr, t = 6 hr, t = 8 hr and cells were count to determine how fast they multiply. The cell cultures reached saturation between 8 hr-9 hr so t = 8 hr was decided to be the last timepoint to follow. For the cell size analysis, the cells from the O/N cultures were collected at the fast growing phase, fixated with gluteraldehyde (as explained in DAPI and calcofluor staining below) and observed under the microscope. Independent samples t-test was performed using the SPSS version 22 package to compare wild type cells and mutants for statistically significant differences in growth rate at different timepoints and cell size. Shapiro-Wilk test was also performed for each sample to prove normal distribution of cell number, as a prerequisite for t-test.

Stress Protocol

Different types of stressors were scanned to understand the role of *caf5⁺* in the cells. Osmotic stress was induced by adding the indicated amount of KCl, CaCl₂ or Sorbitol into the YEA agar. The cells were initially grown O/N at 30°C in YEA broth until they reached 0.5–1 x 10^7 cells/ml. They were then diluted to a total of five serial dilutions, containing 5, 50, $5 × 10^2$, $5 × 10^3$ and $5 × 10^4$ cells (8 μl each) and spotted onto YEA agar media (plain or with KCl, CaCl₂ or Sorbitol). In case of DNA damaging agents, hydroxyurea plates were prepared and the cells were spotted onto agar plates similar to the osmotic stress conditions, as explained above. For UV damage, however, the cells were spotted onto YEA plain media and exposed to different strengths of UV light immediately. The growth was observed after 3-5 days incubation at 30°C.

DAPI and Calcofluor Staining

Schizosaccharomyces pombe cells were initially grown O/N at 30°C in YEA broth until they reached $0.5×10^7$ cells/ml. 1 ml of the cell culture was mixed with gluteraldehyde (2.5% final concentration) and incubated at 4°C for 30 min. After being centrifuged at 3000 rpm for 2 min at 4°C, the cells were re-suspended in 500 μl ice-cold phosphate buffer saline (PBS). They were washed with PBS three times and re-suspended in 20–30 μl ice-cold PBS. 1ml of the sample was either mixed with 1 μl of 100 μg/ml DAPI or calcofluor on the microscope slide to be examined.

Results

The Role of caf5+ in Normal Growth Rate

To understand the significance of *caf5⁺* gene, deletion mutants were formed and scanned in terms of a series of cellular processes. *caf5⁺* deletion was performed as explained in Bähler *et al.* (1998). In this procedure, pFA6a-kanMX6 gene deletion cassette containing kanamycin resistance gene was amplified by specific primers (with complementary sequences to the immediate upstream and downstream regions of *caf5⁺* gene). The product was transformed into the wild type cells to replace *caf5⁺* gene, which is driven by intrinsic homologous recombination mechanisms (Fig. 1A). The mutants were checked by antibiotic resistance for the presence of deletion cassette in the genome and by colony PCR to prove the correct genomic location (the forward and reverse primers are shown as F and R, respectively in Fig. 1A). For *caf5⁺* deletion, the expected PCR product is 305 bp long. Six different colonies that could grow on YEA + G418 were selected for colony PCR. The results showed that the colonies gave positive results for the presence of deletion cassette in the correct location of the genome (Fig. 1B). The fifth colony was chosen for further characterization analysis.

The first experiment was performed to check if *caf5⁺* gene was involved in normal growth at the optimum conditions. *caf5Δ* cells and wild type cells grown at optimum conditions were compared in a time course analysis, where the cell numbers were followed for a total of 8 hours. Samples were collected at every 2 hours and the cell numbers were count. There was no significant

difference between the wild type and *caf5Δ* cells at t = 0 (p = 0.434), t = 2 hr (p = 0.294), t = 4 hr (p = 0.364), t = 6 hr (p = 0.281) time points. However, at t = 8 hr, *caf5Δ* cells were significantly more than wild type cells (p = 0.043). At t = 8 hr, cells approached saturation, so no more time points were collected from this point on (Fig. 1C). The results showed a slightly faster growth in *caf5Δ* cells, which could only be observed in a time course experiment but not on agar plates (Fig. 1C vs. Fig. 2A YEA plate). This result indicates a small but significant role of *caf5⁺* in cell division under optimum conditions, in the absence of cellular stress. Mean ± standard deviation for the wild type cell numbers at different time points were 1.00 ± 0.63 for t = 0 hr, 1.88 ± 0.85 for t = 2 hr, 4.50 ± 2.14 for t = 4 hr, 6.46 ± 1.47 for t = 6 hr, 13.65 ± 6.93 for t = 8 hr. Mean ± standard deviation for the *caf5Δ* cell numbers at different time points were 1.30 ± 0.57 for t = 0 hr, 2.42 ± 0.85 for t = 2 hr, 5.75 ± 2.40 for t = 4 hr, 7.79 ± 2.46 for t = 6 hr, 20.83 ± 3.76 for t = 8 hr. The results showed normal distribution for all the samples (the significant values in Shapiro-Wilk test for the wild type and *caf5Δ* cells at different time points were as follows: wild type (0.053 for t = 0 hr, 0.062 for t = 2 hr, 0.368 for t = 4 hr, 0.258 for t = 6 hr, 0.356 for t = 8 hr); *caf5Δ* (0.228 for t = 0 hr, 0.382 for t = 2 hr, 0.201 for t = 4 hr, 0.579 for t = 6 hr, 0.437 for t = 8 hr).

The Effect of caf5+ Deletion in Cell Size

One very important aspect of life cycle in *S. pombe* is the cell size. In normal growth, *S. pombe* cells are rod shaped and when they reach a critical size, they are expected to divide. Any delay in the cell cycle results in longer cell length and premature progression of cell division is expected to result in shorter cell length. In parallel with this, many cell cycle regulator gene mutations were associated with abnormal cell size. The most extreme examples are mutants of *cdk* regulators: *cdc25* and *wee1* gene deletions in *S. pombe* are well known to have elongated and shorter phenotype, respectively. Therefore, the cell size in *caf5Δ* cells was also checked to see any such cell size defect. The results showed that *caf5Δ* cells were significantly shorter than the wild type cells (p = 0.006) (Fig.. 1D). As discussed above, this result is an indication of involvement of *caf5⁺* gene in cell division control. *Schizosaccharomyces pombe* spermine family transporter *SPBC409.08* gene deletion was previously identified to have a shorter cell size (Güngör & Örs Gevrekci 2016). Double mutants *caf5Δ SPBC409.08Δ* cells were formed and checked for the cell size in the second part. However, no significant difference was found between *caf5Δ* and *caf5Δ SPBC409.08Δ* in terms of cell size (p = 0.160), so the phenotype was not exacerbated in double mutation (Fig. 1D). Mean ± standard deviation of cell sizes (in μm) were 13.39 ± 1.37 for the wild type cells, 12.00 ± 1.65 for *caf5Δ*, and *12.86 ± 2.13* for *caf5Δ SPBC409.08Δ*. The results showed normal distribution for all the samples (0.677 significance in Shapiro-Wilk test for the wild type cells, 0.742 for *caf5Δ*, 0.873 for *caf5Δ SPBC409.08Δ*).
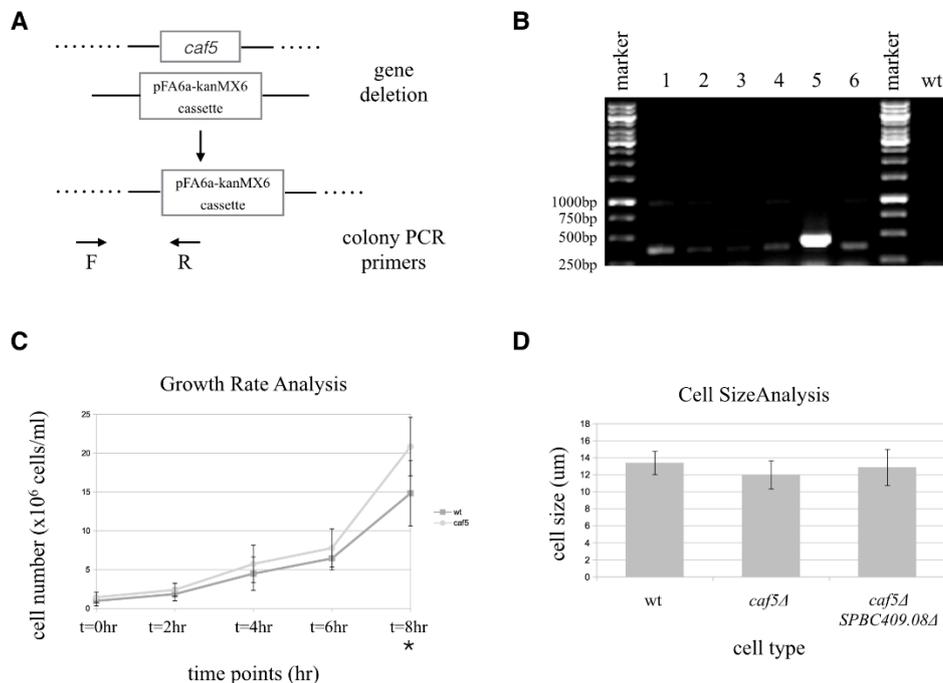

Fig. 1. Schematic representation of gene deletion (A), colony PCR (B), growth rate analysis (C) and cell size analysis of *caf5Δ* (D). (A) A summary of gene deletion and consequent confirmation of deletion by colony PCR, including the forward (F) and reverse (R) primer locations. (B) Both of the colonies (1-6) had the deletion cassette in the correct location of the genome, successfully replacing the *caf5⁺* gene. The expected product size was 305 kb. The colony PCR result for wild type (wt) cells with intact *caf5⁺* is shown as a negative control. (C) Time course experiment graph shows the cell numbers at different time points from t = 0 hr to t = 8 hr. The only timepoint with a significant difference between wild type and *caf5Δ* is indicated with an asterix (t = 8 hr). (D) Cell size comparison of wild type and *caf5Δ* cells. The bars represent standard error.

The role of caf5+ in stress response

One of the most studied and best characterized function of the polyamines is in stress response. At the next step, we checked whether *caf5⁺* was involved in the response against different environmental stressors. Wild type and *caf5Δ* cells were exposed to different kinds of environmental stress such as osmotic stress induced by salts in the media, UV irradiation and hydroxyurea. Hydroxyurea is known to inhibit ribonucleotide reductase (RNR) enzyme and deplete dNTPs, which starves DNA Polymerase for dNTPs. UV irradiation, on the other hand, induces pyrimidine dimers in the DNA. *caf5Δ* cells were plated onto YEA agar plates with high amounts of KCl, CaCl₂ and sorbitol, along with wild type cells to understand any osmotic stress sensitivity. It is noteworthy that different concentrations of KCl, CaCl₂ and sorbitol were tried to see osmotic stress response, increasing gradually starting from lower concentrations up until wild type cells stoped growing. The highest concentrations that allowed normal growth of wild type cells were shown in Fig. 2A. The results showed that *caf5Δ* cells were not different from wild type cells in osmotic stress sensitivity.
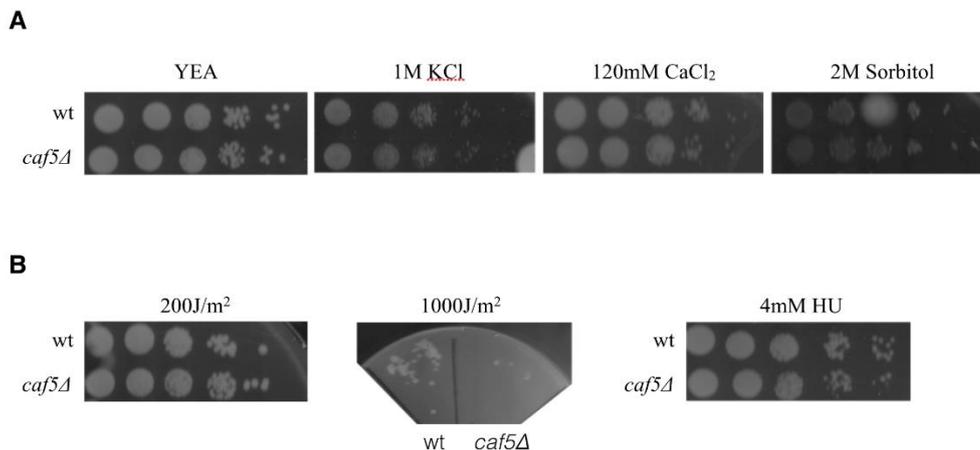
A

B

Fig. 2. Environmental stress response of the *caf5Δ*. Spots on the plates represent 10-fold serial dilutions starting with 5 x 10⁴ cells, 5 x 10³ cells, 5 x 10² cells, 50 cells and 5 cells, from left to right. (A) KCl, CaCl₂ and sorbitol were applied as osmotic stress causers. *caf5Δ* cells showed no sensitivity against osmotic stress. (B) *caf5Δ* and wild type cells were exposed to different strengths of UV irradiation and hydroxyurea. *caf5Δ* cells had reduced viability upon 1000 J/m² UV irradiation but not against hydroxyurea.

Another group of environmental stress factor is DNA damaging agents such as UV irradiation or hydroxyurea. *caf5Δ* cells were exposed to direct UV irradiation and plated onto YEA media to be incubated at the optimum conditions. Similar to osmotic stress, different strengths of UV irradiation was applied. It was shown that *caf5Δ* cells showed no UV irradiation sensitivity between 50-500 J/m² (200 J/m² was shown as a representative in Fig. 2B). However, *caf5Δ* cells grew slightly slower at 1000 J/m² compared to the wild type cells (Fig. 2B). When 1000 J/m² UV light was applied, even the wild type cell growth could be observed well on spot tests, so streak onto plates was preferred to be able to place more cells on the plate and hence for better observation. The next DNA damaging agent checked was hydroxyurea. Exposure to different concentrations of hydroxyurea revealed no sensitivity of *caf5Δ* cells, they grew in a similar pattern with wild type cells (Fig. 2B). The results altogether indicate involvement of *caf5⁺* gene in stress response specifically induced by UV, but not hydroxyurea, KCl, CaCl₂ and sorbitol.

The Mitotic and Meiotic Progression in caf5Δ Cells

Previous research has shown that polyamines were involved in cell cycle regulation and polyamine biosynthesis and transporter gene mutations were associated with defects in cell cycle progression, as explained in the introduction. In *S. pombe,* polyamine deprivation was shown to cause disintegration of the nucleus and absence of septum (Chattopadhyay *et al.* 2002).
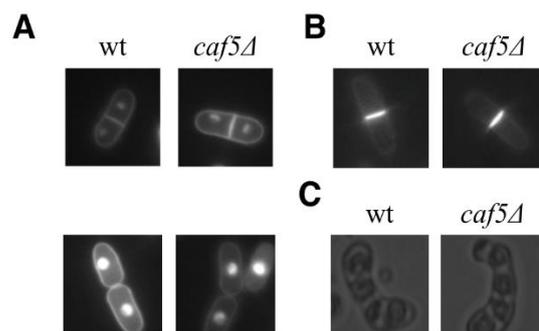

Fig. 3. Cell morphology analysis of *caf5Δ*. (A) Wild type and *caf5Δ* cells were observed under the microscope after DAPI staining to visualize the DNA. (B) Wild type and *caf5Δ* cells were observed under the microscope after calcofluor staining to visualize the septum. (C) Wild type and *caf5Δ* spores formed on the SPA medium

In an attempt to see any cell cycle defects and associated phenotypes, *caf5Δ* and wild type cells growing at optimum conditions were fixated and stained with DAPI and calcofluor to visualize DNA and septum formation. No defective phenotypes in *caf5Δ* cells at

different stages of the cell cycle were seen and the DNA seemed to segregate well in cell division (Fig. 3A). In addition, calcofluor staining showed proper septum formation (Fig. 3B).

Meiotic division was also checked in *caf5Δ* cells by observing spore formation. *caf5Δ* cells were incubated with the cells of the opposite mating type on SPA medium, upon which successful spore formation was observed (Fig. 3C).

Discussion

It is now well established that polyamines are involved in proper cell cycle progression in the cells. Consistent with the role of polyamines in both of these processes, polyamine biosynthesis enzyme ODC level is shown to oscillate during the cell cycle in *S. cerevisiae* (Kay *et al.* 1980), and polyamine depletion from the environment was shown to cause a number of cell cycle dependent phenotypes in *S. pombe* as mentioned before (Chattopadhyay *et al.* 2002). Cell cycle control is also known to be interconnected with stress response pathways. A very important checkpoint in the cell cycle is at the G1 stage, at which cells monitor the extracellular and intracellular environment for nutrients, growth factors, DNA damage etc. In the presence of stressors, cells are programmed to respond to these stress conditions and regulate the initiation of cell division accordingly. Similar to the cell cycle control, stress response is another process that includes polyamine function (Gevrekci, 2017).

Since polyamines contribute to many different functions in the cells including normal cell growth, polyamine levels in the cells are well regulated by redundant mechanisms such as *de novo* biosynthesis, interconversion, cellular uptake and release as well as degradation (Cohen 1998). Polyamine transporters are transmembrane proteins that drive influx and efflux of polyamines. They can be found in the plasma membrane as well as organelle membranes. *Schizosaccharomyces pombe* cells have a number of spermine and spermidine transporter genes with high sequence similarity. They share a major facilitator superfamily (MFS) domain in their protein sequence. Among these genes, *caf5+* codes for a spermine family transporter, which differs from the other spermine and spermidine transporters by not having a sugar transporter domain. This gene previously came up in a scan that showed its overexpression caused resistance to high doses of caffeine (Benko *et al.* 2004). In this study, we aimed to better understand the significance of *caf5+* in different cellular processes in *S. pombe*. Fission yeast *S. pombe* is a haploid organism in its normal cell cycle whose cell cycle defects can be easily observed. *caf5+* deletion mutants were created and scanned for different processes to reveal its involvement in normal growth, cell cycle and stress response, which are the conserved functions of polyamines in different organisms.

caf5Δ cells were very similar to wild type cells in many aspects such as their normal growth at optimum conditions. The viability and growth rate was not reduced in the absence of *caf5+*, but a slightly faster growth could be observed at 8 hr timepoint, just before saturation in *caf5Δ* cell culture. This pattern of growth could be the result of a slightly faster cell division in *caf5Δ* cells. To better focus on the premature/faster cell cycle progression phenotype, the next experiment was designed to compare the cell size of *caf5Δ* and wild type cells. The cell size in *S. pombe* is a good indicator of premature or delayed cell division, as in the case of *wee1Δ* and *cdc25Δ* cells. The results showed that *caf5Δ* cells were shorter than the wild type cells. When slightly faster growth rate and shorter cell size phenotypes were considered together, it can be interpreted that *caf5Δ* caused earlier cell cycle initiation compared to wild type cells. This might be related to the function of polyamines in stress response and their potential to slow down the cell cycle initiation according to the environmental stress (Krüger *et al.* 2013, Gevrekci 2017).

Despite the potential effect of *caf5+* on cell cycle progression, *caf5Δ* cells showed no extreme abnormal phenotypes such as cut or nuc phenotypes or lack of septum formation. cut and nuc phenotypes are mostly induced upon anaphase defect in *S. pombe* that are seen in APC/C conditional mutants (Hirano *et al.* 1986). It is also noteworthy that in case of these phenotypes the cells could not go any further in the cell cycle and the cells are not viable. In case of *caf5Δ* cells, DAPI and calcofluor staining not only ruled out this possibility but also helped to visualize successful progression of different stages in the total population. In addition to the mitotic division, meiotic progression is also scanned by observing spore formation. *Schizosaccharomyces pombe* cells, which are haploid in normal life cycle, are known to arrest at G1 upon nutrient starvation, then mate with the opposite mating type to form diploid cells and finally form spores by meiotic division. Previous researches showed that a very low number of gene mutations (1% of the *S. pombe* genes) cause sporulation defects in *S. pombe*, unlike *S. cerevisiae* (Ucisik-Akkaya *et al.* 2014). For instance, *spo4* and *spo6* were the two genes whose mutations caused failure in the formation of four spores in sporulation media (Nakamura *et al.* 2000, 2002). *caf5Δ* cells were also checked for the presence of sporulation defect but they could successfully form four spores in SPA sporulation media, indicating proper meiosis.

Considering the significance of polyamines in resistance to environmental stressors, polyamine transporters are expected to contribute to stress response at least indirectly. Wild type and *caf5Δ* cells were exposed to different kinds of osmotic stress and DNA damaging agents to understand if loss of *caf5+* causes any sensitivity to stress. Osmotic stress was induced by KCl, CaCl₂ and sorbitol and *caf5Δ* cells could grow as well as wild type cells in the presence of osmotic stress. In case of DNA damaging agents, hydroxyurea and UV irradiation was used. The results showed that at high doses of UV irradiation, the viability of the *caf5Δ* cells was reduced,

but hydroxyurea did not cause a similar effect. The DNA damage caused by the UV irradiation is by the formation of dimers in the DNA, which halts the cell cycle at G1 or G2/M transition. In case of hydroxyurea, however, dNTPs are depleted, DNA replication slows down and cells cannot pass through G2/M transition. The results indicate the significance of *caf5+* particularly in the UV-induced DNA damage response. The results of the stress response, in combination with cell size and growth rate analysis, might show the role of *caf5+* gene in the regulation of the cell cycle regulation upon environmental stress. It can be speculated that *caf5+* gene activity at G1 delays the initiation of cell division to give cells enough time for DNA repair and deletion of *caf5+* could to some extend accelerate cell cycle. It is crucial to note that further research is needed to understand the cell cycle dependent activity of the transporters and their specific roles.

One of the biggest challenges of working with genes involved in polyamine transport is the fact that polyamine levels are kept under strict control by redundant mechanisms. Thus, lack of one transporter is compensated to some extent by other mechanisms. This is the reason for that deletion of polyamine transporter genes are viable at optimum conditions and might have weaker phenotypes. We have previously observed that *S. pombe* cells with mutations in spermine family transporter *SPBC409.08* only had a deviation in cell size with normal cell growth (Güngör & Örs Gevrekci 2016). A similar result was observed for *caf5+,* which showed small but consistent variation in cell size and cell growth. A previous study also showed that spermine family transporter SPBC36.01c was shorter in length compared to the wild type cells, as observed in *caf5+Δ* cells (Örs Gevrekci 2017). When the present and previous studies are considered together, another common phenotype between spermine family transporters and *caf5+* turns out to be the fact that both deletion mutants showed

sensitivity to UV. It is, however, notable that unlike spermine family transporters, *caf5+* deletion did not induce any sensitivity to hydroxyurea (Örs Gevrekci 2017). Altogether, it can be concluded that polyamine transporters are involved in cell size control and DNA damage responses as redundant mechanisms. It is crucial to keep in mind that some of the phenotypes are shared between certain spermine and spermidine transporters, while each of the polyamine transporters can have unique contributions as shown by differing phenotypes at certain cellular processes.

In addition to the transporters, biosynthetic and degradation enzymes contribute to the polyamine homeostasis. Previous studies also revealed the interplay between synthesis, degradation and transport by showing that increase in polyamine levels negatively regulate polyamine synthesis and uptake in mammalian cells (Gesteland *et al.* 1999, Rom & Kahana 1994). A previous study on *S. pombe* polyamines showed that prolonged polyamine depletion from the environment was shown to induce a number of cell cycle dependent phenotypes (Chattopadhyay *et al.* 2002). This proves the significance of polyamines for the cells. The same study also showed that even a small amount of polyamine could restore normal cell growth and division. So, in summary, although they are involved in multiple crucial processes, cells have evolved different mechanisms for polyamine homeostasis, which in turn makes it harder to identify and characterize transporters/biosynthetic/degradation genes hard. As a future extension, combination of different mutants could reveal activatory and inhibitory pathways in polyamine homeostasis.

Acknowledgement

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