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FUNCTIONAL INTERACTION BETWEEN *caf5* AND SPERMIDINE FAMILY TRANSPORTERS IN *S. POMBE*

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ABSTRACT

Polyamines are well known for their wide range of roles in the cells, including stabilization of nucleic acids, regulation of gene expression at different levels, stress response and cell cycle. The activity of these well conserved small polycations are also regulated by different mechanisms to assure their intracellular concentration and activity. Polyamine transporters have a major role in the regulation of the influx and efflux of the polyamines. Previously, two spermine family transporters *SPBC409.08* and *caf5* were characterized which were associated with cell size defects. The aim of this study is to better understand the role of *caf5* plasma membrane spermine transporter and its functional interaction with spermidine transporters. Double mutants of *caf5* and spermidine family transporters *SPBC36.01c*, *SPBC36.03c* and *SPBC569.05c* were formed in *S. pombe*. The double mutants were then put through growth rate analysis, meiotic progression and sporulation analysis, cell cycle analysis, and vulnerability to environmental stress. The results showed that the double mutants differentiate from each other in their combined mutation with *caf5* in terms of cell size and DNA damage response, indicating redundant seperate pathways of spermine and spermidine transport in *S. pombe*.

Keywords: *S. pombe*, Cell Size, Cell Cycle, Stress Response, Polyamines,

1. INTRODUCTION

Polyamines are small, ubiquitous molecules that are very well conserved among different organisms. They are aliphatic hydrocarbon chains with amine groups, which exist as polycations at the physiologic pH. They are known to exist in different forms, the most common of which are spermines and spermidines.

From small microorganisms to higher eukaryotes, polyamines seem to take on very important roles in the cells. Due to their polycationic nature, they can readily bind to nucleic acids DNA and RNA, and thus stabilize them [1]. Polyamines' contribution to the integrity of DNA and RNA is known to be especially important for the survival of thermophilic microorganisms (thermostability) in extreme temperatures [2]. Polyamines are also involved in the regulation of gene expression, at the level of translation, by binding to RNA complexes in bacteria and yeast [3-5]. A better characterized and one of first identified function of the polyamines is to promote stress response in a wide range of organisms including yeast, fungi and plants. Polyamines are known to regulate stress response against temperature, oxidative and osmotic stress [6]. Therefore, any disruption in the polyamine homeostasis is associated with vulnerability to different kinds of environmental stress.

The significant roles polyamines overtake in the cells necessitate strict regulation of their homeostasis by different mechanisms such as regulation of intracellular biosynthesis, molecular degradation and cellular transport in and out of the cell [7]. Polyamine biosynthesis can be regulated by different pathways, the most common of which initiates with the conversion of l-ornithine into putrescine by ornithine decarboxylase (ODC). Putrescine is then converted to spermidine by spermidine synthase (SpdSyn) and consequitively spermidine can be converted into spermine by spermine synthase (SpmSyn). Previous

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studies have shown that mutations in the genes that are involved in polyamine biosynthetic pathway result in growth rate decrease in the presence of stress or decreased pathogenicity [8, 9].

A less characterized pathway for the polyamine homeostasis is the transmembrane transport of spermine and spermidine via specialized transmembrane molecules on the plasma and organelle membranes. The activity of transporters are crucial for the organisms who lack biosynthesis enzymes and rely on the uptake of environmental polyamines [10]. The activity of the transporters are subject to environment-dependent regulation resulting in reduced polyamine export out of the cell in response to environmental growth stimuli [11, 12]. Additionally transporters turned out to be promising drug targets in bacteria [13, 14] due to their contribution in the survival of the organisms.

Despite the fact that polyamines regulate crucial functions, a very small number of research has been conducted on these molecules. One reason for the small number of research about polyamine transporters is the high number of redundant transporters, which can take over each others function. In *S. pombe* for instance deletion mutants of individual polyamine transporters are all viable and shows a weak phenotype of environmental stress vulnaribility and cell cycle defects. Moreover, deletion of different transporters showed vulnerability to different kinds of environmental stressors (such as hydroxyurea or UV irradiation), which indicates their specialized differential roles at different stages of cell cycle [15, 16]. It is also noteworthy that the transporters are especially important in the presence of any secondary mutation in the biosynthesis genes, in case of which organisms depend on the uptake of environmental polyamines.

This study aims to uncover the functional interaction between spermine and spermidine family transporters in *S. pombe*. Previous research has shown that although defective phenotypes could be observed in single spermine and spermidine deletions, they were quite weak phenotypes and the cells stayed viable at optimum conditions. This is a strong indication that alternative, redundant pathways exist in the cells that cover up for the loss of single polyamine transporter gene. Therefore it is crucial to understand the functional interaction between these genes. To this end, single mutants of spermine family transporter *caf5* were combined with previously characterized spermidine family transporters and scanned for different functions. It is hypothesized that genes that are involved in seperate pathways would exacerbate or reverse certain phenotypes when mutated together depending on the activatory or inhibitory effect.

2. MATERIAL AND METHOD

2.1. Model Organism and Media

Schizosaccharomyces pombe strain 972 (*h- ade*) was used to make single mutations of *caf5* gene and also as wild type control. The other mutant strains were formed in *S. pombe* strain 975 (*h+ ade*) background. Both the wild type cells, single and double mutants were handled as explained in [17].

Two different media is used in the experiments. First one is the YEA medium, which includes 5 g/L Difco yeast extract, 30 g/L glucose, 75 mg/L adenine, pH adjusted to 5.6 with HCl. Agar YEA media is formed by adding 2% agar (w/v). The media is supported by different concentrations of salt to induce stress response just before sterilization. For the spot tests in the absence and presence of osmotic stress, the cells were initially grown to a density of 0.5-1x10^7 cells/ml and then spotted on the agar media in tenfold serial dilutions (starting from 5 × 10^4 cells until 5 cells), to be incubated at optimum conditions (30 °C for 3–5 days).

The second media used is the low nitrogen SPA media that includes 1 g/L KH2PO4, 10 g/L glucose, 1 mL/L 10,000X vitamin stock solution (10 g/L pantothenate, 100 g/L nicotinic acid, 100 g/L inositol, 100 mg/L biotin). The low nitrogen media promotes G1 arrest and consequitive meiotic division in *S. pombe,* which is followed by sporulation in the presence of opposite mating type. The cells were streaked on SPA supplemented with 2% agar (w/v) and incubated for 5 days at 25 °C to induce sporulation and mating.

2.2. Forming Double Mutants in *S. pombe*

To form double mutants *h-* and *h+* strains of single mutants (which also carry different antibiotic resistance genes, either kanamycin or hygromycin as well as different mutations) were streaked together on SPA agar medium to be incubated at 30 °C for 3 days. The cells were then initially replica-plated onto YEA+300µg/ml hygromycin to be incubated for 3 days at 30 °C to select for the presence of hygromycin resistance gene and hence the first mutation. Then, the cells were replica-plated onto YEA+ 200µg/ml G418 to select for the presence of kanamycin resistance gene and hence the secondary mutation.

2.3. Stress Protocol

In order to reveal any defect in the stress response, *S. pombe* cells were initially grown in 50 mL of YEA medium, O/N at 30 °C until they reached 0.5×10^7 cells/mL. The cells were then diluted and plated onto YEA agar supplemented with either 0.5M KCl, 0.2 M NaCl, 120mM $CaCl_2$ or 2 M sorbitol depending on the test as explained above. The reason for the use of these specific concentrations of KCl, NaCl, $CaCl_2$ or sorbitol is the fact that these are the maximum concentrations that we could see proper growth by spot tests [15, 16]. To check for the DNA damage response against UV-irradiation, however, the cells were plated onto YEA agar plates and then exposed to UV irradiation right away. The cells were finally incubated at 30 °C for 3–5 days.

3. RESULTS

3.1. Growth Rate Analysis at the Optimum Conditions and Under Environmental Stress

caf5 deleted cells were previously shown to have normal vegetative growth under optimum conditions and under environmental osmotic stress [18]. To see the effect of secondary spermidine family transprter gene mutations and hence functional interactions in growth rate, the growth of double mutants were observed on agar plates. As shown in Figure 1A, on plain YEA agar, double mutants grew at a similar pattern to the wild type cells, without any delay.

The next analysis was performed to uncover the growth rate of the double mutants in the presence of DNA damaging agent – UV irradiation. Different strengths of UV light was applied to the double mutants starting from 50 J/m^2 to 1000 J/m^2. For the sake of simplicity only 200 J/m^2 and 1000 J/m^2 was shown in Figure 1B. It is important to note that when 1000 J/m^2 UV irradiation was applied the number of cells spotted was not enough to see survival. Therefore we used streak test, rather than spot test, to observe the growth of even the wild type cells. This phenomenon was previously discussed [16]. As shown in Figure 1B, although no phenotype was observed at 200 J/m^2, the cells had a slower growth rate at 1000 J/m^2. However, notably, double mutants' growth rate had slight but consistent variation. The wild type cells could survive well when exposed to 1000 J/m^2 and could form colonies from single cells as can be seen by colonies around the midline. In case of *caf5Δ,* however, independent single colonies are significantly reduced around the midline. Number of single colonies around the midline was also reduced in *caf5Δ SPBC36.01cΔ* and *caf5Δ SPBC36.03cΔ* mutants, but in case of *caf5Δ SPCC569.05cΔ* single colonies could be seen around the midline. In summary, The growth of *caf5Δ SPCC569.05cΔ* showed a similar pattern to wild type cells, while *caf5Δ SPBC36.01cΔ* and *caf5Δ SPBC36.03cΔ* mutants grew slightly slower than the wild type cells as detected by the appearance of individual colonies around the midline.

It was also checked if double mutants showed any sensitivity to osmotics stress, which was induced by adding KCl, NaCl, $CaCl_2$ or sorbitol in YEA agar plates. The results showed that double gene mutations did not result in sensitivity to osmotic stressors (Figure 2).

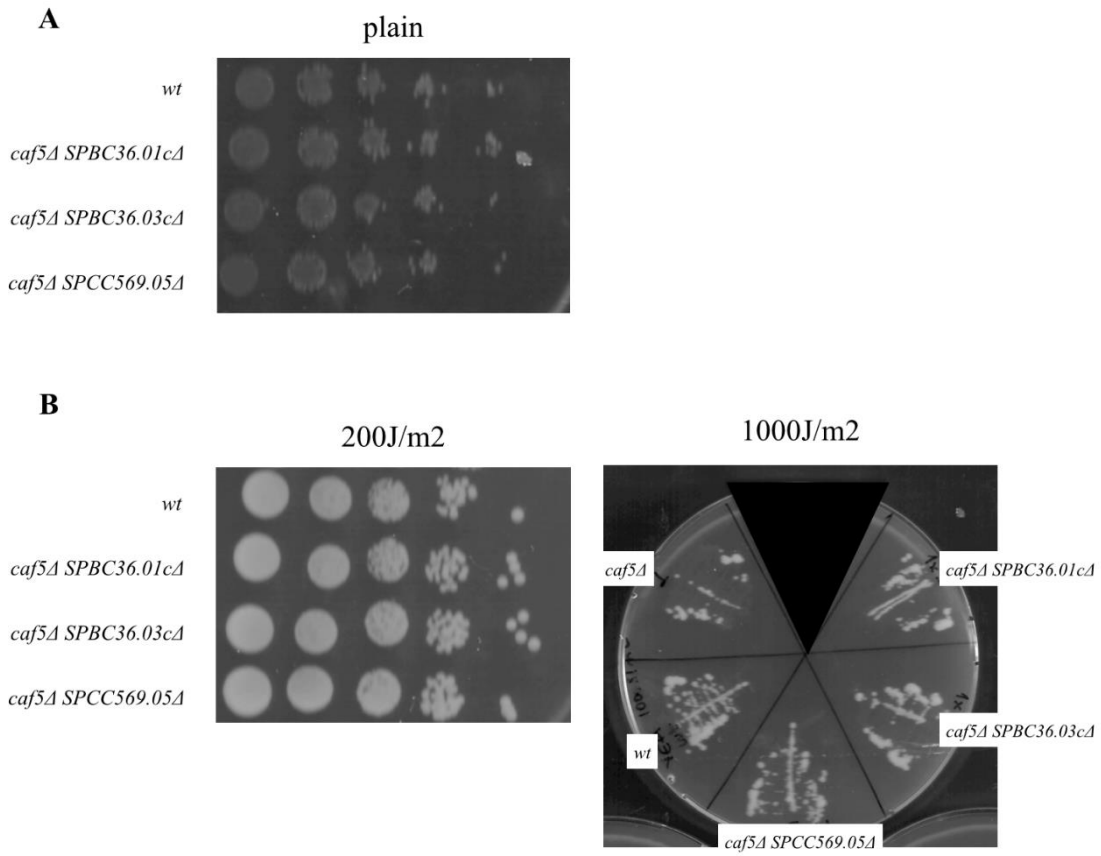

Figure 1. Growth rate analysis at the optimum conditions and in the presence of DNA damaging agents as an environmental stress. (A) Wild-type, *caf5Δ SPBC36.01cΔ, caf5Δ SPBC36.03cΔ* and *caf5Δ SPCC 569.05cΔ* cells were grown at the optimum conditions, on YEA plain agar media at 30°C. (B) Wild-type, *caf5Δ SPBC36.01cΔ, caf5Δ SPBC36.03cΔ* and *caf5Δ SPCC 569.05cΔ* cells were exposed to 200 and 1000J/m^2 UV irradiation immediately after being spotted or streaked on the YEA plain agar media and incubated at 30°C.

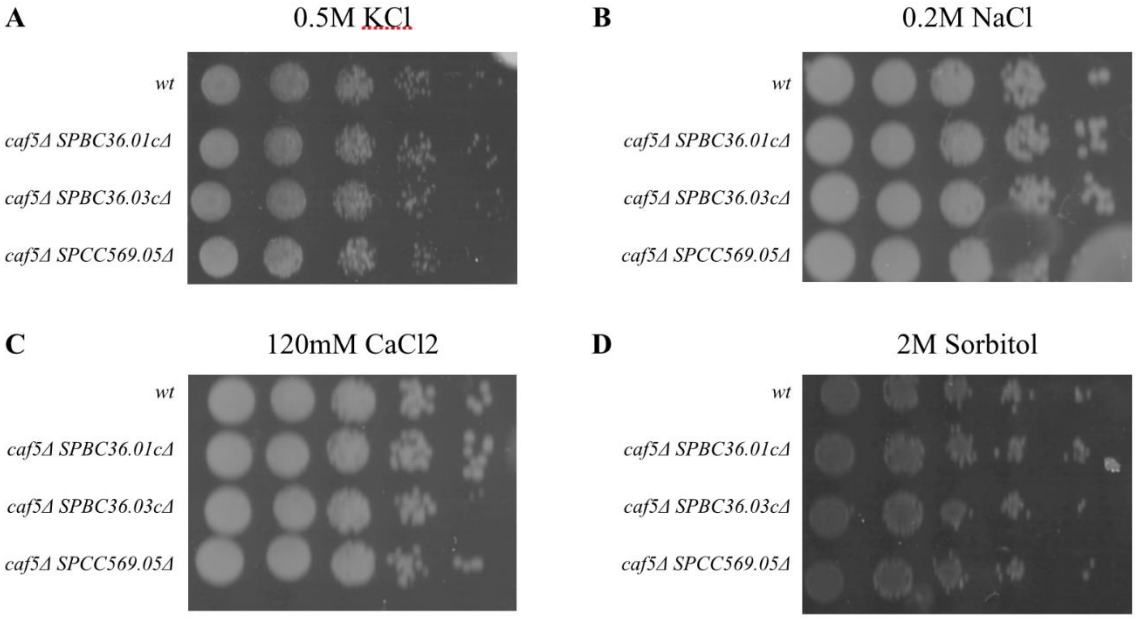

Figure 2. Osmotic stress response of the *caf5Δ SPBC36.01cΔ, caf5Δ SPBC36.03cΔ* and *caf5Δ SPCC 569.05cΔ* cells in comparison to wild-type cells. Stress response of the mutants were induced by the YEA agar media supplemented with (A) 0.5M KCl, (B) 0.2M NaCl, (C) 120mM $CaCl_2$ and (D) 2M sorbitol.

3.2. The Effect of Polyamine Transporters on Meiotic Progression

In order to understand any defect in the meiotic progression, sporulation of the wild type and double mutants were induced by SPA media and examined for the sporulation. Formation of four successful spores would indicate successful induction of G1 arrest upon nitrogen deprivation, mating and spore formation by meiosis. The results showed that both the wild type and double mutants could form spores when incubated on SPA sporulation media (Figure 3).

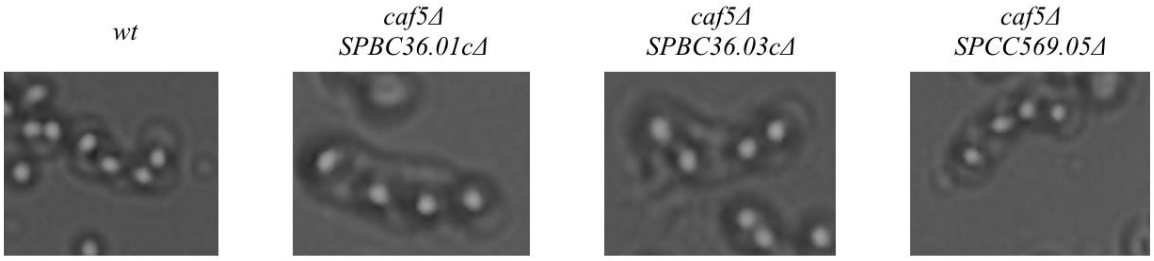

Figure 3. Spore formation in the wild-type, *caf5Δ SPBC36.01cΔ, caf5Δ SPBC36.03cΔ* and *caf5Δ SPCC 569.05cΔ* as an indicator of proper G1 arrest, mating and meiotic progression.

3.3. The Functional Interaction between *caf5* and Spermidine Family Transporters in the Regulation of Cell Size

The wild type and double mutants were analyzed in terms of cell size, as an indicator of cell cycle progression. Spermine family transporter gene mutant *caf5Δ* cells was previously found to be significantly shorter than the wild type cells [18] and the spermidine family transporter gene mutants *SPBC 36.01cΔ*, *SPBC 36.03cΔ* and *SPCC 569.05cΔ* were previously shown to be similar to the wild type cells in terms of cell size [15]. Independent samples t-test was used to compare the cell size of the wild type cells and double mutants. In case of the double mutants, however, *caf5Δ SPBC36.01cΔ* cells and *caf5Δ SPBC36.03cΔ* cells showed a similar phenotype to the *caf5Δ* cells, while *caf5Δ SPCC 569.05cΔ* double mutant's cell size was similar to the wild type cells. The double mutants *caf5Δ SPBC36.01cΔ* ($p<.032$) and *caf5Δ SPBC36.03cΔ* ($p<.047$) were significantly shorter than the wild type cells, while the other double mutant *caf5Δ SPCC569.05cΔ* ($p<.783$) showed no significant difference to the wild type cells, indicating that a secondary *PCC569.05c* gene deletion reverted shorter the phenotype caused by the *caf5* deletion (Figure 4). The mean values ± standard deviation is as follows: wt (13.40±1.37); *caf5Δ SPBC36.01cΔ* (12.27±1.79); *caf5Δ SPBC36.03cΔ* (13.27±1.36); *caf5Δ SPCC569.05cΔ* (15.09±3.44).

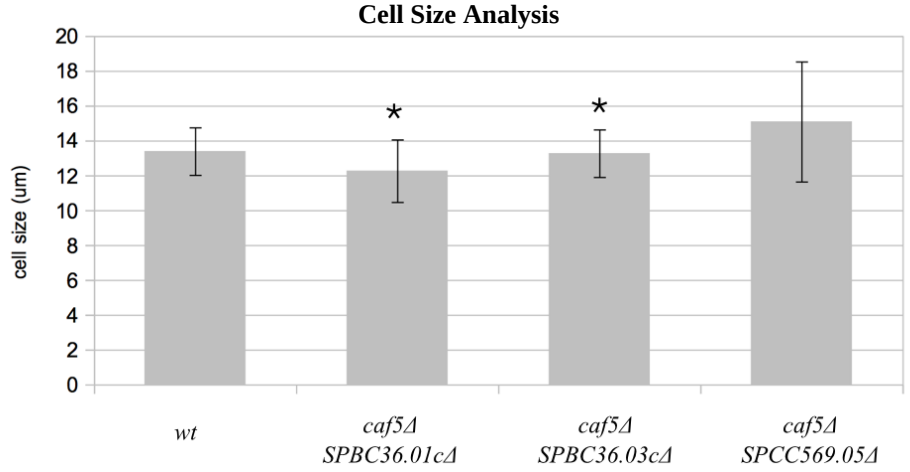

Figure 4. Cell size analysis of the wild-type, *caf5Δ SPBC36.01cΔ, caf5Δ SPBC36.03cΔ* and *caf5Δ SPCC 569.05cΔ* cells. The double mutants that are significantly different than wild type cells in terms of cell size are shown with an asterisks. The *p* values are as follows: for *caf5Δ SPBC36.01cΔ* $p<.032$, for *caf5Δ SPBC36.03cΔ* $p<.047$ and for *caf5Δ SPCC569.05cΔ* $p<.783$.

4. DISCUSSION

Among a high number of ubiquitous molecules, polyamines have a special role since they are involved in many different functions in the cells. Polyamines can be either synthesized *de novo* or imported from the environment. Therefore, environmental intake of polyamines by polyamine family transporters (spermine or spermidine transporters) contribute significantly to polyamine homeostasis. In case of a dysregulation in polyamine biosynthesis, cells rely on transport of polyamines from the environment for viability [10]. Polyamine transport also exists in the opposite direction, transfering polyamines to the environment. In summary both influx and efflux of polyamines, particularly spermine and spermidine, is provided by transmembrane transporters. In addition to the plasma membrane, these transporters can also exist in the organelle membranes.

Having a high number of genes dedicated to the transport of polyamines provide redundant mechanisms that simultaneously regulate polyamine concentrations in the cells. In research, however, this makes the characterization of these genes more difficult since deletion/mutation of single transporter genes can be at least partially covered up by other transporter genes and the mutants show off a weak phenotype. So understanding the function of individual transporter genes requires simultaneous mutations in multiple genes. This could also shed light into the differential functional interactions between the transporter genes, which can be grouped in different pathways. In this study different deletion mutants were combined to understand the differential functional interactions between spermine and spermidine genes. To this end, spermine transporter *caf5Δ* was combined with spermidine family transporters *SPBC36.01cΔ, SPBC36.03cΔ* and *SPCC569.05cΔ.*

Polyamines were known to be involved in multiple functions in *S. pombe.* They include DNA damage response, regulation of cell cycle and stress response [15, 16, 19]. Consistent with this deletion mutants of polyamine biosynthesis genes, polyamine modification enzymes and polyamine transporters resulted in cell cycle defects, vulnerability to DNA damaging agents and deviations in the cell size. For instance *caf*5*Δ, SPBC36.01cΔ* and *SPBC36.03cΔ* are both sensitive to DNA damaging agents [15, 18] while such a phenotype could not be observed in *SPCC569.05cΔ* [15]. All of these genes are transmembrane polyamine transporters with a high sequence similarity. They both have a transmembrane domain as well as a MFS (major facilitator superfamily) domain, which is known to determine the site of substrate transport [20]. Forming double mutants and observing whether the phenotypes of the single mutants are either exacerbated or reduced will shed light into the functional interaction between these genes. Such that if the two genes are in the inhibitory pathways double mutants are expected to reduce the phenotype. If on the other hand the genes are working in the activatory pathways, mutual deletion of these genes are expected to enhance the phenotype.

The results showed that double mutants have functional meiosis just like the single mutants. So meiotic progression could be successfully followed in the absence of two of the polyamines. A similar effect was detected in the stress response against osmotic stressors (KCl, NaCl, $CaCl_2$ and sorbitol). These results showed that intracellular polyamines that are synthesized de novo or by interconversion is enough to run G1 arrest, mating and meiosis. So spores could be formed successfully even in double mutants. Also stress response against high levels of salt could be tolerated in double mutants. A very interesting result was observed when the double mutants were analyzed in terms of DNA damage response and cell size.

The single deletion of *SPBC36.01c, SPBC36.03c* and *SPCC569.05c* were both similar in cell size to the wild type cells but *caf5Δ* cells were significantly shorter. The cell size in *S. pombe* goes beyond being a good indicator of proper cell growth and it also indicates proper cell cycle control. The *cdk* regulators *wee1* and *cdc25* gene mutations are the best examples for this, *wee1* being shorter and *cdc25* being longer than wild type cells. When the double mutants were analyzed in terms of cell size it was found that double mutants *caf5Δ SPBC36.01cΔ* and *caf5Δ SPBC36.03cΔ* inherited caf5*Δ* shorter cell size. Interestingly *caf5Δ SPCC569.05cΔ* double mutants were similar to wild type cells in terms of cell size.

This shows that a secondary *SPCC569.05c* deletion overrides the *caf5* deletion phenotype. This is an indication that *SPCC569.05* gene might work in an inhibitory pathway to *caf5*. Another result supporting this idea came from DNA damage analysis. When exposed to high energy UV light, *caf5* and S*PBC36.01c* genes were already known to be important for the survival. For *SPBC36.03c* no such sensitivity to UV light was detected previously. It is also important to mention that *SPBC36.01cΔ* and *SPBC36.03cΔ* cells were both sensitive to another DNA damaging agent, hydroxyurea. Therefore it is not surprising that *caf5Δ SPBC36.01cΔ* and *caf5Δ SPBC36.03cΔ* cells also showed some level of sensitivity to UV irradiation. In case of *caf5Δ SPCC569.05cΔ* cells, however, UV sensitivity is almost diminished as shown by the appearance of single colonies around midline. To sum up, in case of DNA damage sensitivity, cell size phenemenon was repeated.

To summarize, despite the sequence similarity and being specialized in spermidine transport, spermidine family transporters *SPBC36.01c, SPBC36.03c* and *SPCC569.05c* differentiate from each other in terms of their interaction with spermine family transporter *caf5*. Interaction between spermine and spermidine transporters are expected to serve and important funciton since interconversion of different polyamines contribute greatly to the intracellular amounts of these multifunctional molecules.

Our results are consistent with the previous studies, which showed that polyamine transporter genes are involved in mitosis, cell morphology and stress response, especially DNA damage response [11, 12, 15, 16]. More specifically, our result that *caf5* mutation can have a different cell size and DNA damage response phenotype when combined with *SPCC569.05c* mutation is in the same line with a previous study that showed double gene mutations (of polyamine transporters and/or biosynthesis enzymes) can come up with different phenotypes [10]. Additionally, the fact that a secondary *SPBC36.01c* or *SPBC36.03c* mutation did not change the *caf5* phenotype supports the idea that polyamine transport is run by redundant pathways, which might also have specific functions.

Similar to polyamine transporters, polyamine synthase molecules have significant contributions to polyamine metabolism. So as a future direction, single and double mutants of polyamine synthase genes and transporter genes will be analyzed. Understanding the regulation of polyamine function will help us to better understand the regulation of cell cycle, stress response, gene expression and finally how they are interconnected with each other. Growing number of research has accumulated recently about the use of polyamines in drug design against pathogens [13, 14] or in nanotechnology [21]. This also necessitates better understanding of the polyamines and polyamine regulators in different organisms.

5. CONCLUSION

The presence of multiple genes with similar function serves a lot of function in the cells. The first one is to secure redundant pathways in case any one of these genes are missing. The second one is to provide multiple regulatory steps either inhibitory or excitatory. The results obtained in this study shows that multiple polyamine family transporters, specialized in spermine or spermidine transport, interact with each other in different ways. Both *SPBC36.01c, SPBC36.03c* and *SPCC569.05c* are specialized in spermidine transport with a high sequence similarity, however, this study shows that *SPCC569.05c* differs from the other two in terms of its interaction with *caf5* spermine family transporter. The conclusion that should be delivered from this study is spermine and spermidine transporters are functionally interconnected with each other and spermidine transporters differentiate from each other in their regulatory activity.

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