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The role of predicted spermidine family transporters in stress response and cell cycle in *Schizosaccharomyces pombe*

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Abstract: Fission yeast *Schizosaccharomyces pombe* has a variety of stress-signaling proteins that protect cells against environmental or intracellular stress. These proteins help the cells to respond to stress conditions and regulate intracellular functions such as cell division or gene expression. Polyamines (spermidine, spermine, and putrescine) are known to be important in the regulation of stress response and cell division. In this study, we tried to experimentally characterize novel *S. pombe* genes that are involved in the polyamine pathway and understand their potential roles. Sequence analysis revealed four genes that code for (predicted) spermidine family transporters in *S. pombe*. In an attempt to characterize these (predicted) spermidine family transmembrane transporters and their possible roles, deletion mutants of these candidate genes were created. These mutants were exposed to different stress conditions, such as DNA-damaging agents and osmotic stress, to understand their significance in the stress response. Next, the mutants were analyzed in terms of cell size, growth rate, and spore formation to understand their contribution to cell cycle control. The results revealed that individual deletion of two of these genes, *SPBC36.01c* and *SPBC36.02c*, resulted in sensitivity to DNA-damaging agents, indicating their role in DNA damage response.

Key words: Polyamines, spermidine, *Schizosaccharomyces pombe*, cell cycle, stress response

1. Introduction

Polyamines (spermidine, spermine, and putrescine) are small organic molecules with amine groups that are well conserved in different organisms. They exist in all living species except the Archaea, Methanobacteriales, and Halobacteriales (Hamana and Matsuzaki, 1992). They are involved in multiple functions in the cells, such as cell growth, differentiation, cell cycle, apoptosis, stress response, and cell survival (Schipper et al., 2000; Wallace et al., 2003; Liu et al., 2007). At physiological pH polyamines are positively charged, which enables their interaction with DNA. Spermidine and spermine form a bridge between the minor and major grooves of DNA and influence the structure and function of the DNA (Hampel et al., 1991; Roberts et al., 1992; Matthews et al., 1993), which is the reason why polyamine depletion results in partial unwinding of DNA. They can also regulate transcription of genes such as *c-myc* (Celano et al., 1992).

Polyamines are mostly studied in plants and they are known to be especially important in surviving stress conditions, such as UV irradiation or drought (Kusano et al., 2007; Liu et al., 2007). As shown in a number of studies, polyamines are also involved in cell cycle regulation in multiple cells; hence, depletion of polyamines or inhibition of the biosynthetic pathway leads to cell cycle defects (Fredlund and Oredsson, 1996; Ray et al., 1996; Choi et al., 2000; Igarashi and Kashiwagi, 2010). For instance, in fission yeast *Schizosaccharomyces pombe*, lack of spermine and spermidine leads to cell cycle defects (Russell et al., 1975). Due to their extensive effects on cell survival, polyamine inhibitors are even thought to be candidate agents in the prevention of cancer (Casero and Marton, 2007; Nowotarski et al., 2013). Consistent with this, there is also evidence that polyamine overproduction and polyamine metabolism dysregulation are associated with many different forms of cancer and their metastatic properties (Casero and Marton, 2007; Soda, 2011).

Since polyamines are involved in so many processes, polyamine metabolism is strictly regulated in the cells (Cohen, 1998). Redundant mechanisms have evolved to ensure polyamine homeostasis, such as regulation of polyamine synthesis and transport of polyamines into and out of the cells by specific transporters. Polyamine transport is sensitive to the growth rate of cells. Polyamine export out of the cell is turned on by decreased growth rate and turned off in response to growth stimuli, such as

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serum and nutrients (Wallace and Keir, 1981; Wallace and Mackarel, 1998). There are further feedback mechanisms to control polyamine levels in the cell such that increased polyamine levels lead to an increase in antienzyme 1 in mammalian cells, which negatively regulates the synthesis and uptake of polyamines (Rom and Kahana, 1994; Gesteland et al., 1999). This negative feedback loop keeps the polyamine levels constant in cells. A similar negative feedback loop also exists for *P. polycephalum*, *S. cerevisiae*, *N. crassa*, and *S. pombe* (Mitchell and Wilson, 1983; Williams et al., 1992; Toth and Coffino, 1999; Ivanov et al., 2000).

The *S. pombe* genome has been completely sequenced and there are a number of genes that await experimental characterization. Four of these genes, *SPBC36.01c*, *SPBC36.02c*, *SPBC530.15c*, and *SPCC569.05c*, are named as predicted spermidine family transmembrane transporters based on sequence similarity. They share major facilitator superfamily domains. The *S. pombe* database PomBase was used to obtain the sequences of these uncharacterized/predicted spermidine family transporters. There are very limited experimental data on these uncharacterized genes. In a microarray-based study, cells that are made cisplatin-resistant upon chronic exposure to the drug showed decreased *SPCC569.05c* gene expression (Gatti et al., 2004). Another study showed that in a genome-wide scan, SPBC36.01c protein tagged with GFP was localized on the membrane. This is consistent with the transmembrane segment sequence, which is predicted in sequence analysis. This study tried to further characterize four of the predicted spermidine family transporters and reveal their potential roles in stress response and cell cycle regulation.

2. Materials and methods

2.1. Yeast strains and media

The methods for handling *S. pombe* were as described by Moreno et al. (1991). Strains used in this study were 972 (h^- *ade*) and 975 (h^+ *ade*). YEA medium (5 g/L Difco yeast extract, 30 g/L glucose, 75 mg/L adenine, pH adjusted to 5.6 with HCl) was used to incubate cells. Agar medium contained 2% agar (w/v). Tenfold serial dilutions (starting from 5×10^4 cells until 5 cells) were spotted out on the medium in aliquots of 8 µL and incubated at 30 °C for 3–5 days. To induce sporulation, the cells were then incubated on SPA low-nitrogen medium (1 g/L KH_2PO4, 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)) for 5 days at 25 °C.

2.2. Gene deletion in *S. pombe*

A PCR-based gene targeting method (Bähler et al., 1998) was used for constructing gene deletion. pFA6a-kanMX6 (Bähler et al., 1998) and pFA6a-hphMX6 (Sato et al., 2005) plasmids carrying kanamycin and hygromycin resistance genes, respectively, were used to replace the gene that would be deleted. The cells were initially incubated in a medium containing 200 mg/L G418 to select for the kanamycin resistance gene and 300 µg/mL hygromycin to select for the hygromycin resistance gene. The cells that could survive on selective media with antibiotics were checked with colony PCR according to whether they had the antibiotic resistance cassette in the right place.

2.3. Confirmation of the mutants by colony PCR

Colony PCR was performed to make sure the deletion cassette was integrated into the correct location. The forward primer was designed for the cassette and the reverse primer for the downstream region of the gene. The colonies were initially boiled with the dNTP mix, PCR buffer, and primers at 98 °C for 10 min. Taq polymerase was added after the mixture had been cooled down. The PCR program was as follows: 30 cycles at 94 °C for 20 s, 50 °C for 40 s, and 72 °C for 1 min/kb.

Deletion mutants of (predicted) spermidine family transporter genes are as follows (mating type, antibiotic cassette): *SPBC36.01c* (h^+, hygromycin), *SPBC36.02c* (h^-, kanamycin), *SPBC530.15c* (h^-, kanamycin), *SPCC569.05c* (h^+, hygromycin).

2.4. Lithium acetate (LiOAc) method of transformation in *S. pombe*

S. pombe cells were grown in 100 mL of YEA medium, O/N at 30 °C until they reached 0.5–1 × 10^7 cells/mL. The cells were then centrifuged down at 4000 rpm for 2 min. The pellet was washed with 50 mL of ddH_2O and then with 1 mL of 0.1 M LiOAc (pH 4.9). After washing, cells were incubated in 2 mL of 0.1 M LiOAc (pH 4.9) at RT for 1 h. Following incubation, cells were centrifuged down at 4000 rpm for 2 min. The pellet was resuspended in 150 µL of 0.1 M LiOAc (pH 4.9) and incubated with 100 ng of DNA (the deletion cassettes) and 350 µL of 50% polyethylene glycol for 1 h at RT. Following incubation, 58 µL of DMSO was added and the cells were heat-shocked at 42 °C for 5 min. The cells were allowed to cool to RT for 10 min and centrifuged at 4000 rpm for 2 min. The pellet was washed with 1 mL of ddH_2O and recentrifuged. Finally, the pellet was resuspended in 100 µL of ddH_2O and plated on the agar medium with the nutritional supplement (YEA). After an incubation period of 24 h at 30 °C, the cells were replica-plated onto selective agar medium with the antibiotics.

2.5. 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. They were then plated onto YEA agar containing 4 mM hydroxyurea, 0.5 M KCl, 0.2 M NaCl, or 2 M sorbitol. The cells were plated onto the corresponding agar in 10-fold serial dilutions starting from 5 × 10^4 cells until 5 cells. For the UV-dependent DNA damage response, however, the cells were plated onto YEA agar plates and they were exposed to UV irradiation right away. The cells were then incubated at 30 °C for 3–5 days.

3. Results

3.1. Verification of deletion mutants

Predicted spermine family transporters were selected from the PomBase website (http://www.pombase.org). Based on sequence similarity, *SPBC36.01c*, *SPBC36.02c*, *SPBC530.15c*, and *SPCC569.05c* were identified as predicted spermidine family transporters in PomBase. To characterize these genes experimentally, deletion mutants were generated and verified by first planting the cells on antibiotic-containing media and then colony PCR.

3.2. Growth rate analysis

The deletion mutants were first compared to the wild-type cells in terms of growth rate at the optimum conditions (YEA agar plate, 30 °C). A time course experiment was conducted to find out any defect in cell growth. To this end, wild-type and deletion mutant cultures were prepared at a density of 10^6 cells/mL and cell numbers were analyzed every 2 h. The cell numbers were compared using a two-tailed Student's t-test and no significant difference was observed between the doubling times of the wild-type cells and any of the deletion mutants. The results are shown in Figure 1. To summarize, under optimum conditions, the mutants grow as well as the wild-type cells.

3.3. Cell size analysis

As a second step in the experimental characterization of the mutants, the cell sizes of all the deletion mutants were compared to wild-type controls. Previous work has shown that cell size could be an indicator of a cell cycle progression defect and abnormal cell size could be a result of a cell cycle regulator gene mutation. For instance, *wee1* deletion results in shorter cell length, while *cdc25* deletion results in elongated cells in *S. pombe*. Thus, in an attempt to understand the potential role of spermidine family transporters in cell cycle progression, the mutant and wild-type cells were grown in YEA liquid medium at 30 °C until they reached the log phase. The cells were analyzed under the microscope during their fast-growing phase to determine cell length. For each gene deletion and the control group, 40 individual cells were observed and statistically analyzed in this assay. A two-tailed Student's t-test was performed to detect any statistically significant differences between the wild-type cells and the deletion mutants. Figure 2 shows the mean cell size values of the cells. The Table summarizes the mean values of the cell sizes, standard deviations (mean ± STD), 95% confidence interval upper and lower bounds, and P-values (computed in the 95% confidence interval). The results showed that the predicted spermidine family transporter *SPBC36.01cΔ* has a shorter cell size compared to wild-type cells.

3.4. Sporulation defect analysis

The *S. pombe* cell cycle is very sensitive to environmental conditions, which are mainly monitored at the G1 phase of the cell cycle. The cells are known to arrest at the G1

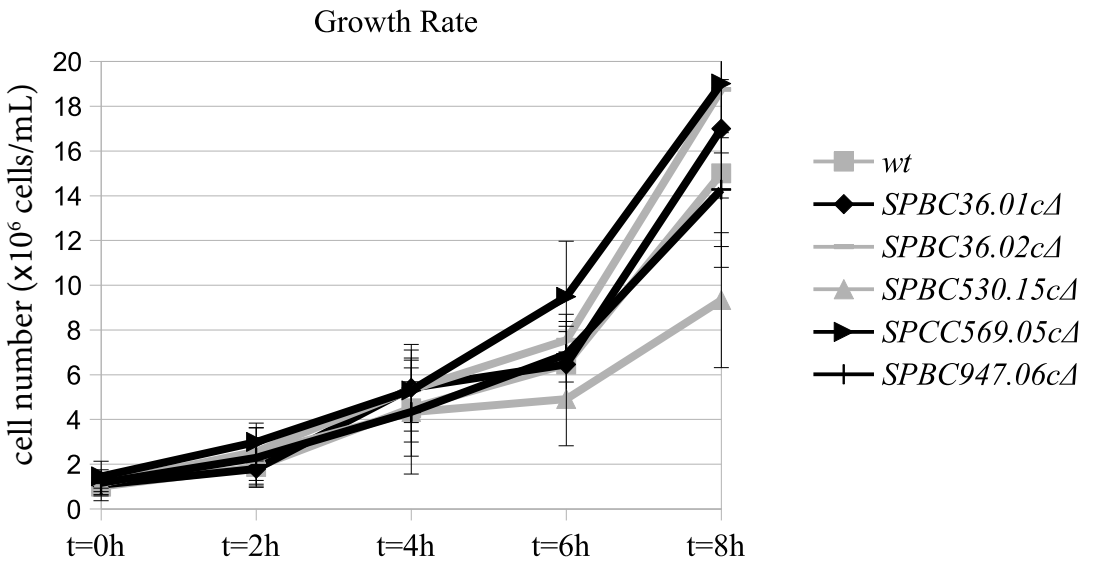

Figure 1. Growth rate analysis of the wild-type (*wt*), *SPBC36.01cΔ*, *SPBC36.02cΔ*, *SPBC530.15cΔ*, and *SPCC569.05cΔ* cells under optimum conditions.

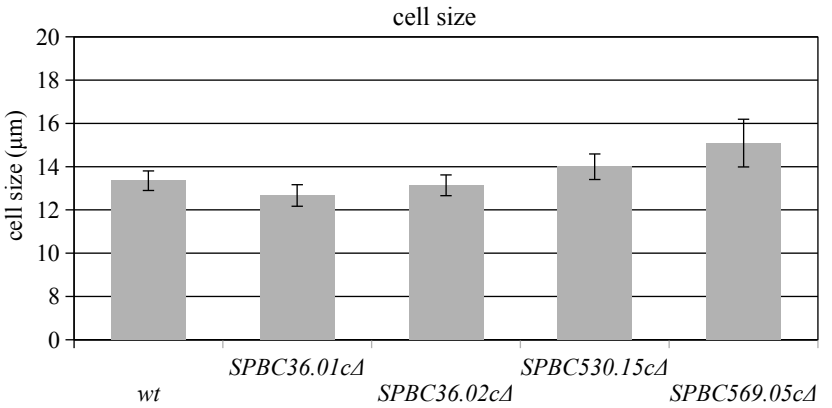

Figure 2. Cell size analysis of the wild-type, *SPBC36.01cΔ*, *SPBC36.02cΔ*, *SPBC530.15cΔ*, and *SPCC569.05cΔ* cells. Error bars represent 95% confidence intervals.

Table. Cell size analysis.

Name of the gene	Mean cell size (µm) ± STD		P-value
	95% confidence interval	Lower bound	
		Upper bound	
wt	13.35 ± 1.42	12.90	-
		13.81	
SPBC36.01cΔ	12.66 ± 1.56	12.17	0.04
		13.16	
SPBC36.02cΔ	13.14 ± 1.50	12.66	0.63
		13.61	
SPBC530.15cΔ	14.00 ± 1.83	13.41	0.34
		14.59	
SPCC569.05cΔ	15.09 ± 3.44	13.98	0.58
		16.19	

phase in the case of environmental stress conditions, such as nutrient starvation. In this study, the nitrogen from the media (SPA medium) was depleted to induce G1 arrest. Upon G1 arrest, the wild-type cells mate with the cells of the opposite mating type, form diploid cells, and then go through meiosis to create spores. In this step, the deletion mutants were mixed with the wild-type cells of the opposite mating type and spore formation on SPA medium was observed after 3–5 days of incubation. No sporulation defect was observed in any of the four mutants (Figure 3A). The number of sporulations in each culture was also checked, as shown in Figure 3B. The two-tailed Student's t-test was performed to detect any statistically significant differences between the wild-type cells and the deletion mutants and no significant difference was observed. P-values computed in the 95% confidence interval were as follows: *SPBC36.01cΔ* ($P < 0.51$), *SPBC36.02cΔ* ($P < 0.74$), *SPBC530.15cΔ* ($P < 0.75$), and *SPBC569.05cΔ* ($P < 0.55$).

3.5. Stress response analysis

Polyamines such as spermidine are well known to be involved in the stress response. Hence, we decided to check if the deletion of (hypothetical) spermidine family

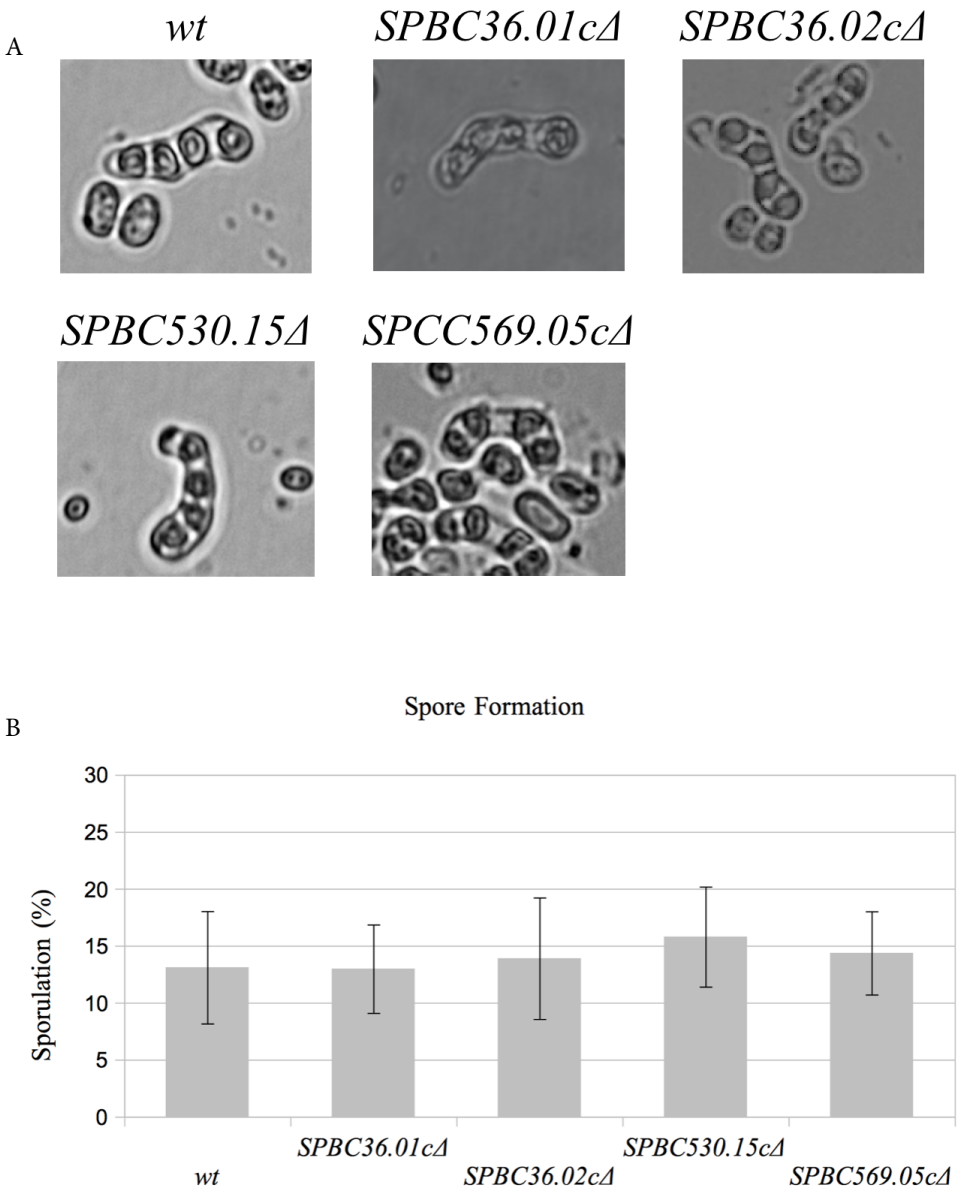

Figure 3. Spore formation in the wild-type (*wt*), *SPBC36.01cΔ*, *SPBC36.02cΔ*, *SPBC530.15cΔ*, and *SPCC569.05cΔ* cells (A) and sporulation efficiencies of the *wt* and mutant cells (B). Error bars represent 95% confidence intervals.

transporter genes has any effect on the stress response. Two different types of stress were applied to the mutants as well as wild-type control cells. The first type of stress applied was environmental osmotic stress. Osmotic stress was induced by preparing YEA agar plates containing different amounts of KCl, NaCl, or sorbitol. Conditions (0.5 M KCl, 0.2 M NaCl, and 2 M sorbitol) are shown in Figure 4. As seen in Figure 4, under osmotic stress conditions, the deletion mutants did not show decreased viability. Higher concentrations of KCl (1 M), NaCl (1 M), and sorbitol (4 M) were used so that even wild-type cells stopped growing, but no difference between the growth rates of wild-type cells and the mutants was detected. The second type of stress was induced by DNA-damaging agents. Two types of DNA damage were induced. The first was UV irradiation, which causes dimer formation in the DNA molecule, and the second one was hydroxyurea, which depletes dNTPs and stops DNA replication in the S phase. The same principle as osmotic stress was applied to DNA-damaging agents: UV exposure and hydroxyurea were increased until the wild-type cells stopped growing. In the case of hydroxyurea, two of the mutants, *SPBC36.01cΔ* and

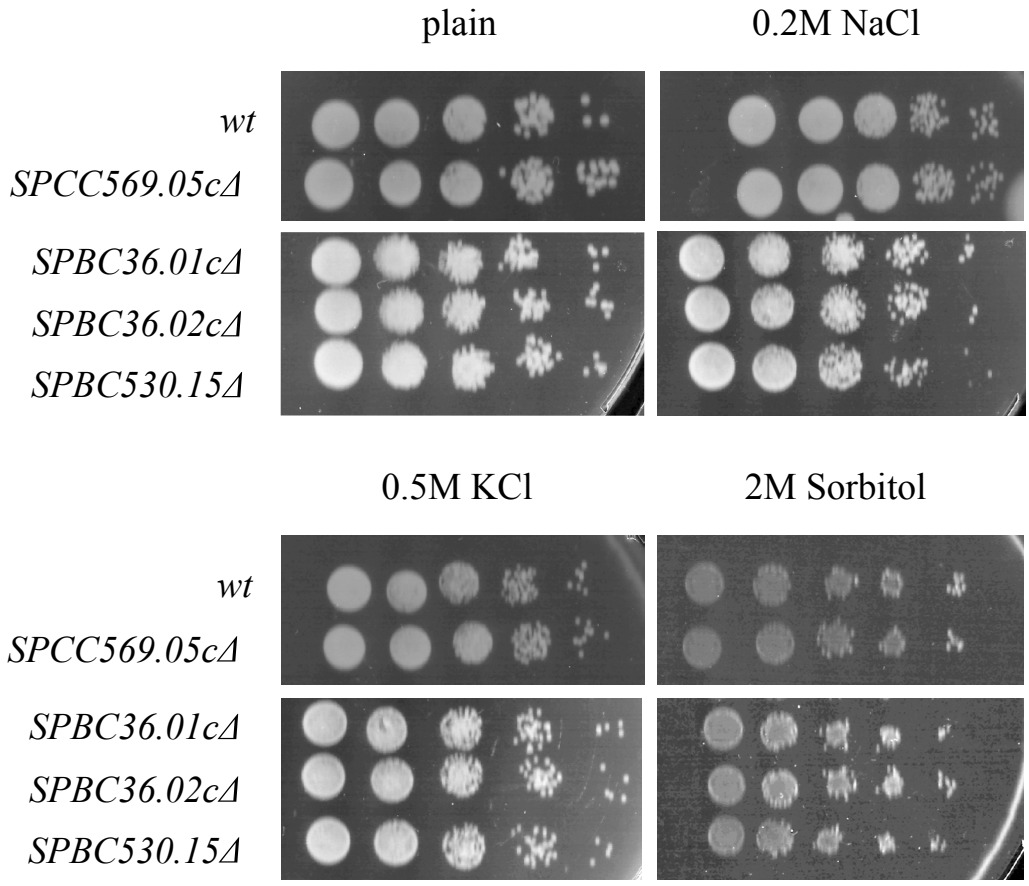

Figure 4. Osmotic stress response of the mutants compared to wild-type cells (*wt*), under different concentrations of 0.2 M NaCl, 0.5 M KCl, and 2 M sorbitol.

SPBC36.02c*Δ*, showed some sensitivity so that the cells grew less than the wild-type cells (Figure 5A). In the case of UV irradiation, however, out of the five mutants only *SPBC36.01cΔ* showed sensitivity (Figure 5B).

4. Discussion

Monitoring and responding to environmental and intracellular stress is crucial for the survival of cells. The cell cycle, along with many other intracellular metabolic pathways, is regulated according to the nature of the stress and thus there are proteins that connect these two processes in the cells (Petersen and Hagan, 2005; Ors et al., 2009; Majumdar et al., 2012). Polyamines have long been known for their roles in cell cycle regulation and stress response in different organisms. They are also reported to be involved in apoptosis and gene expression (Schipper et al., 2000; Krüger et al., 2013). Since polyamines are so important in many different processes, polyamine metabolism is kept under strict regulation in the cells. Interconversion and de novo synthesis of polyamines as well as their uptake by specific transporters contribute to the regulation of polyamine metabolism.

S. pombe is one of these organisms in which polyamines are important for proper cell cycle progression. In a previous study, *SPBC409.08* (predicted spermine family transporter) was involved in the cell cycle and *SPAC9.02c* (predicted polyamine N-acetyltransferase) was involved in the DNA damage response (Güngör and Örs Gevrekci, 2016). This encouraged us to examine predicted polyamine transmembrane transporters further. In this study, four candidate genes were identified, which confer sequence similarity to spermidine family transporters: *SPBC36.01c*, *SPBC36.02c*, *SPBC530.15c*, and *SPCC569.05c*. Deletion mutants of these genes were analyzed to reveal potential roles in cell cycle and stress response.

SPBC530.15c and *SPCC569.05c* gene deletions showed similar phenotypes with the wild-type cells in our analysis. There was no significant difference in cell size. They could properly form spores, which indicates that mating and meiosis could proceed successfully in these mutants. When exposed to stress conditions such as increased salt concentration, UV irradiation, or hydroxyurea treatment, these mutants were no more sensitive than the wild-type cells. One explanation for that could be the fact

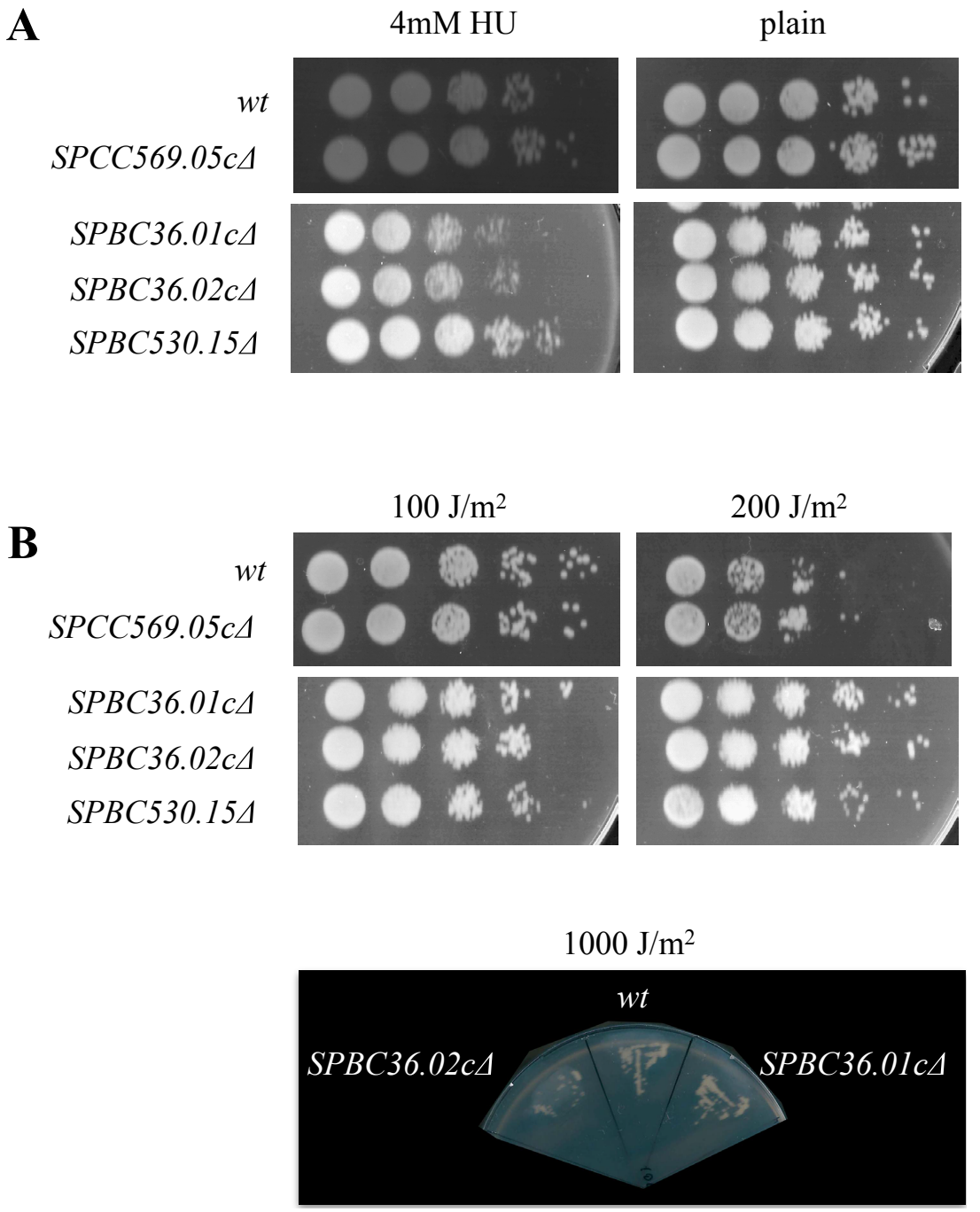

Figure 5. DNA damage response of the mutants compared to wild-type (*wt*) cells upon (A) hydroxyurea administration and (B) different exposures of UV irradiation.

that redundant mechanisms might exist for polyamine transport, so the effect is minimized.

One important effect was detected in *SPBC36.01c* deletion. The cell size of this mutant was shorter compared to wild-type cells, which was a small but statistically significant effect. The short cell size could be an indication of premature cell division, as in the case of *wee1Δ* (Nurse, 1975; Thuriaux et al., 1978). *SPBC36.01cΔ* cells were also detected to be sensitive to UV irradiation at very high exposures (1000 J/m^2). The survival of the *SPBC36.01cΔ* cells was also less than that of wild-type cells in the presence of hydroxyurea, which was a small but consistent effect. *SPBC36.02c* gene deletion was also slightly sensitive to hydroxyurea treatment. These results indicated a

potential role of the *SPBC36.01c* and *SPBC36.02c* genes in DNA damage response. A similar but much stronger effect was observed with the *rad24* gene in *S. pombe*. *rad24Δ* cells were reported to be involved in the DNA damage checkpoint and they also entered mitosis prematurely (Ford et al., 1994), similar to *SPBC36.01cΔ*. The fact that *SPBC36.01cΔ* and *SPBC36.02cΔ* cells were sensitive to DNA-damaging agents is consistent with previous research, which showed that polyamines were involved in plant response to UV irradiation (Kusano et al., 2007). Polyamine synthesis and uptake is negatively regulated by antienzyme 1 upon increased polyamine levels (Gesteland et al., 1999; Niiranen et al., 2002). This negative feedback mechanism keeps the polyamine levels relatively constant in the cells. Consistent with this observation, an increase in polyamine levels could only be observed in the absence of antienzyme 1 (Ivanov et al., 2000). This phenomenon could be the reason why *SPBC36.01cΔ* and *SPBC36.02cΔ* mutants result in mild phenotypes in cell size or DNA damage sensitivity.

Polyamine transporters are involved in polyamine transfer in and out of the cell, regulate intracellular polyamine levels, and consequently contribute to stress response and cell cycle regulation. For instance, budding yeast polyamine transporter Tpo1 provides polyamine uptake at alkaline pH levels and induces polyamine export at acidic pH levels (Uemura et al., 2004). It exports polyamines upon stress and extends cell cycle arrest induced by oxidants (Krüger et al., 2013). This study also showed that polyamine transporters are potential regulators of stress response and cell cycle.

To our knowledge, this study is the first experimental characterization of the *SPBC36.01c* and *SPBC36.02c* genes. These data extend the understanding of the polyamine metabolism players and roles in the cells. Polyamines have been of great interest due to their contribution to cancer progression and metastasis and their potential as therapeutic targets in human (Casero and Marton, 2007). Future extensions of this research involve a better understanding of the protein products of the *SPBC36.01c* and *SPBC36.02c* genes and their interaction partners and regulators. Any protein that physically interacts with the protein products of these genes reveals the potential regulators and highlights the pathways they contribute to. Therefore, tagged versions of these proteins can be used to hunt for physically interacting partners. Considering the wide range of activities that polyamines are involved in, we hope to find proteins related to other metabolic functions besides stress response and cell cycle to investigate as novel interacting partners.

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