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Small heat-shock protein Hsp9 has dual functions in stress adaptation and stress-induced G2-M checkpoint regulation via Cdc25 inactivation in *Schizosaccharomyces pombe*

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ABSTRACT

The small heat-shock protein Hsp9 from *Schizosaccharomyces pombe* was previously reported to be a homologue of *Saccharomyces cerevisiae* HSP12. Although Hsp9 is expressed in response to heat shock and nutritional limitation, its function is still not completely understood. Here, we explored the biological function of Hsp9 in *S. pombe*. The *hsp9* gene might play a role in stress adaptation; *hsp9* deletion caused heat sensitivity and overexpression induced heat tolerance. In addition, Hsp9 also contribute to cell cycle regulation in the nucleus. Δ*hsp9* cells grew more quickly and were shorter in length than wild-type cells. Moreover, Δ*hsp9* cells did not achieve checkpoint arrest under stress conditions, leading to cell death, and exhibited a short doubling time and short G2 phase. Overexpression of *hsp9* induced cell cycle delay, increased the population of G2 phase cells, and rescued the phenotypes of *cdc2-33*, *cdc25-22*, Δ*rad24*, and Δ*rad25* mutants, suggesting that Hsp9 probably regulates Cdc2 phosphorylation by modulating the Cdc25 activity. Indeed, immunoprecipitation experiments revealed that Hsp9 is associated with 14-3-3 and Cdc25. In Δ*hsp9* cells, the association of 14-3-3 with Cdc25 was weakened and Cdc2 phosphorylaton was reduced. Together, our data suggest that Hsp9 has dual functions in stress adaptation and regulating a G2-M checkpoint by the Cdc25 inactivation; this differs from *S. cerevisiae* HSP12, which maintains cell membrane stability under stress conditions.

1. Introduction

Cells from virtually all organisms respond to a variety of stresses by rapid synthesis of a highly conserved set of polypeptides termed heat-shock proteins (HSPs). HSPs are classified into seven major families according to their size: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, Hsp33, and the small HSPs, including the α-crystallins (12–43 kDa) [1,2]. Large HSPs are highly conserved in species of bacteria, yeast, and mammals, but small HSPs are a ubiquitous but diverse class of proteins. Only certain short-sequence motifs of small HSPs, located in the C-terminus (α-crystallin domains) or in the N-terminus (hsp9/12 domain), are conserved and the functions of small HSPs have relatively understudied [3,4]. In higher eukaryotes, some small HSPs are up-regulated by various stresses and are developmentally regulated [5–7]. The small HSPs are also involved in a variety of cellular processes related to cytoskeletal rearrangements [8,9], fibril formation [10], and apoptosis [11,12]. The expression of some small HSPs is increased in neurodegenerative disorders [13] and in certain tumors [14]. Despite their critical roles in the cell, the mechanisms underlying the functions of small HSPs are not well known.

In *Saccharomyces cerevisiae*, a small heat-shock protein HSP12 is strongly induced in response to various stresses, including heat shock or osmotic stress [15–17]. However, few studies have demonstrated a correlation between HSP12 deletion/overexpression and a sensitivity/resistance phenotype, making it difficult to assign a role for HSP12 under various stresses. Recently, it was suggested that HSP12 protects the membrane against desiccation [17] and maintains cell membrane stability under stress conditions [18,19]. Small heat-shock protein Hsp9 of *Schizosaccharomyces pombe* was previously reported as a homologue of *S. cerevisiae* HSP12 [20,21]. Hsp9 may be one of the actin-associated proteins that stabilize the F-actin contractile ring structure [21].

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Although Hsp9 is reportedly induced in response to heat shock and nutritional limitation [20], its function is still not completely understood. In this study, we explored the biological function of Hsp9. We propose that the Hsp9 of *S. pombe* may be crucial for stress adaptation. Hsp9 is also indispensable for G2-M checkpoint control by the Cdc25 inactivation under stress conditions through modulation of the association between 14-3-3 and Cdc25.

2. Materials and methods

2.1. Strains, media and chemicals

S. pombe strains used in this study represented in Table 1. Strains were grown in YES (0.5% yeast extract, 0.5% peptone, and 3% glucose) and EMM (0.3% potassium phthalate, 0.22% Na_2HPO_4, 0.5% NH_4Cl, 2% glucose) containing nutritional supplements as necessary. The standard yeast genetic techniques were performed as described [22].

2.2. Gene disruption and hsp9 gene cloning

For gene deletion, the 5′ and 3′ flanking regions were PCR-amplified with the following primers: sense, 5′- CC*GAATTC*CTGTATTACA TCATAAGAAAGTAG-3′; antisense, 5′- CC*GGATCC*GACAATTAAATTGA TTGGAATTGA-3′; sense, 5'-GCC*GTCGAC*GGCTGGTGAAACTCAATC-CAC-3'; antisense, 5'-CCG*AAGCTT*CCCAAGTCACAACTACTAACT-3', and inserted into the pTZ18U containing the *ura4*$^+$ gene. The open reading frame of gene was replaced with the *ura4*$^+$ gene by gene replacement [22].

To construct *hsp9* expression plasmids, the expression vectors pREP1 and pSLF175 regulated by the *nmt*1 promoter was used [23]. The *hsp9* DNA fragments were PCR-amplified with the following primers: sense, 5′-CC*GGATCC*ATGTCTGATCCCGCAAGAAAG-3′; antisense, 5′-GGG*CCCGGG*TTACAACTTGTCATCAACAA-3′, and ligated into the corresponding sites of the expression vectors.

2.3. Preparation and analysis of synchronous culture

Cells were synchronized by centrifugal elutriation by using a JE-5.0 elutriation rotor (Beckman Coulter, Fullerton, CA) at 2500 rpm [24]. The synchronous elutriated culture was collected by centrifugation at 10 min after the completion of elutriation and resuspended in minimal media. Aliquots were removed at 15-min intervals. A half of the aliquots were fixed in formaldehyde and the proportion of mitotic cells was scored. The others were prepared for immunoblot analysis.

2.4. Hsp9 overexpression and stress-tolerance experiments

S. pombe cells were grown in EMM at 30 °C to mid-exponential phase (OD600 = 0.5). To verify the effects of hsp9 overexpression, cells harboring pREP1 or pREP1-hsp9 were cultivated in EMM containing thiamine. Then, the cells were shifted into fresh thiamine-deficient EMM at OD600 0.1, cultivated to OD600 0.25 at 30 °C, spotted onto agar plates (5 μL of serial 5-fold dilutions), and incubated under different temperature conditions. After 2–3 days, cell survival was determined. *S. pombe* ts mutants were first grown at 23 °C. Cells harboring the empty pREP1 plasmid were used as a negative control. Cells were exposed to UV light from a XL-1000 UV crosslinker (Spectronics Co., NY, USA).

Table 1
Strains used in this study.

Strains	Genotype		
972	*h-*		Lab. collection
ED005	*h-ade6-216 leu1-32*		This study
HD046	*h-ade6-216 leu1-32 ura4-D18*	*hsp9::Ura4*	This study
ED668	*h-ade6-216 leu1-32 ura4-D18*		Lab. collection
HD042	*h-ade6-216 leu1-32 ura4-D18*	*rad24::kanMX4*	This study
HD044	*h-ade6-216 leu1-32 ura4-D18*	*rad25::kanMX4*	This study
ED011	*h-leu1-32*		This study
ED108	*h-cdc2-33 leu1-32*		P. Fantes
ED524	*h-cdc25-22 leu1-32*		P. Fantes
ED511	*h-wee1-50 leu1-32*		P. Fantes

2.5. Microscopy

All fluorescent images were taken with a Leica fluorescence microscope equipped with a high performance CCD camera (Sensicam) and Slidebook software (Intelligent Imaging System). Nuclei and septa of the cells were counter-stained with 4,6-diamine-2-phenylindole-dihydrochloride (DAPI) and Calcofluor, respectively [23].

2.6. Immunoblot analysis and immunoprecipitation

Whole cell extracts were collected and analyzed by immunoblot analysis as previously described [23]. Cells were harvested by centrifugation and washed once with ice-cold washing buffer. The cell pellet was resuspended in lysis buffer, glass beads were added, and the cells were broken with a bead-beater (Biospec Products., Inc.). Cell extracts (200 μg) and 2 μg of antibody against 14-3-3 (Cell signaling) or GFP (Santa Cruz Biotech.) were incubated for 3 h at 4 °C for immunoprecipitation. Immune complexes were incubated with protein A/G agarose (Millipore, MA, USA) beads for 2 h. All immuno-precipitants were washed 10 times with ice-cold washing buffer and co-precipitated proteins were analyzed by immunoblot analysis. Horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Millipore) were used for detection according to the manufacturer's instructions.

2.7. Cell fractionation

Extracts were prepared as described by Seibert et al. [25]. Briefly, cells were resuspended with buffer S (1.4 M Sorbitol, 40 mM HEPES (pH 7.2), 0.5 mM $MgCl_2$, 1 mM PMSF, 100 g mL^{-1} Zymolase) and incubated at 30 °C for 40 min. Cells were gently collected by centrifugation, resuspended in 300 μL buffer F (18% Ficoll 400 (w/v), 40 mM HEPES, 0.5 mM $MgCl_2$, 1 mM PMSF, protease inhibitor), and lysed in a homogenizer. The lysates were mixed with 200 μL buffer GF (7% Ficoll 400 (w/v), 20% glycerol, 20 mM HEPES, 0.5 mM $MgCl_2$, protease inhibitor) and centrifuged for 2 min at 8000 rpm. The cytoplasmic fraction (supernatant) and nuclei (pellet) were collected.

3. Results and discussion

3.1. The hsp9 in S. pombe is a stress-responsive small heat shock gene that does not possess an α-crystallin domain

The small heat-shock protein Hsp9 of *S. pombe* was previously reported as a homologue of *S. cerevisiae* HSP12, which does not possess an α-crystallin domain [20,21]. Analysis of the *hsp9* promoter region revealed a single TATA box at position-75 (MatInspetorV2.2). There are three repeats of the stress-responsive CCCCT element (STRE) at −626, −413, and −258 responsible for mediating transcriptional induction by various forms of stress [26] and a consensus sequence of the heat-shock element (HSE) at −44 to −31, consisting of arrays of the 5-bp unit AGAAN arranged as inverted repeats (Fig. 1A) [27]. Consistent with previous reports, the *hsp9*

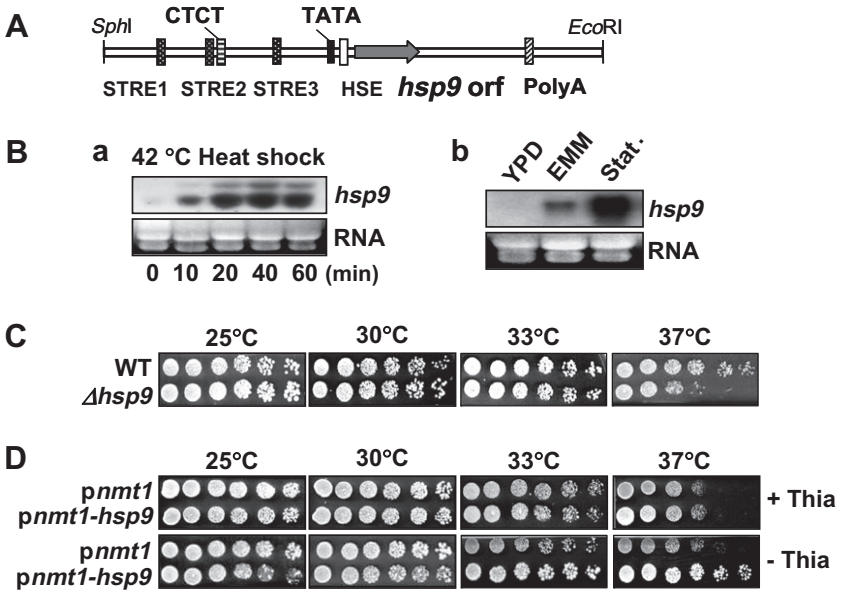

Fig. 1. Hsp9 is responsible for the acquisition of heat tolerance in *S. pombe*. (A) Schematic representation of *hsp9*. The bars represent functional transcription factor binding sites and the designated names of the elements are shown. HSE, putative heat-shock element; STRE, stress-responsive CCCCT motif; TATA, a putative TATA box. (B) Expression analysis of *hsp9*. Total RNAs were isolated from strain 972 (wild-type cells) grown in YES or EMM in the presence and absence of stress. RNA was probed with labeled PCR-amplified *hsp9*-Orf. "RNA" indicates the amount of 18S and 28S RNAs in the same gel stained by ethidium bromide and used as a control. (C) The deletion of *hsp9* enhanced the thermo-sensitivities of *S. pombe* cells. The wild-type cells and Δ*hsp9* mutant were grown, shifted to various temperature conditions as described in "Section 2", incubated for 2–3 days, and survival were monitored. (D) Overexpression of *hsp9* increased the thermo-tolerance of *S. pombe*. The cells containing p*nmt1* or p*nmt1-hsp9* were cultivated in EMM containing thiamine and shifted into fresh thiamine-deficient EMM at OD_{600} 0.1. After growth to OD_{600} 0.25, the cells were spotted onto EMM with or without thiamine and incubated under various stress conditions for 2–3 days.

transcript was induced within 10 min and continued to increase up to 60 min after incubation of the cells at 42°C (Fig. 1B-a). The *hsp9* transcript was minimally detectable in exponentially growing cells, but dramatically induced during stationary phase growth in rich and minimal media (Fig. 1B-b). These results demonstrated that expression of *hsp9* was regulated not only by heat stresses but also by the nutritional depletion signal.

3.2. Hsp9 is responsible for the acquisition of heat tolerance in S. pombe

In order to determine whether Hsp9 is involved in protection against heat stress, the *hsp9* gene was disrupted by gene replacement and the viability of Δ*hsp9* vs. wild-type cells under various temperature conditions was examined. The wild-type and Δ*hsp9* cells were spotted on YES plates after being placed under 25, 30, 33, and 37 °C for 3 h in liquid media and further incubated at respective temperature. The *hsp9* null mutants showed diminished survival at 37 °C in comparison to the wild-type strain, suggesting the deletion of *hsp9* resulted in loss of protection against heat stress (Fig. 1C). To analyze the effect of *hsp9*-overexpression on viability of cells, cells possessing p*nmt1-hsp9* were also spotted on EMM plates with or without thiamine and incubated at 25, 30, 33, and 37 °C (Fig. 1D). The cells overexpressing *hsp9* were resistant to heat stress in comparison to the control strain which carried the empty vector. Therefore, these results suggest that there is a correlation between *hsp9*-deletion or *hsp9*-overexpression and stress-sensitivity or tolerance, and then *hsp9* is responsible for the acquisition of heat tolerance in *S. pombe*.

3.3. Hsp9 accumulates in the nucleus after heat stress and may be involved in the cell cycle checkpoint

To explore the function of Hsp9, we first examined the localization of Hsp9 protein in the presence and absence of stress. Cells carrying p*nmt1-GFP-hsp9* were cultured in EMM media without thiamine and exposed to heat stress at 39 °C (Fig. 2A and B). Microscopy and cell fractionation revealed that the GFP–Hsp9 fusion protein was localized in the cytoplasm and nucleus under non-stressed conditions. The localization of GFP–Hsp9 changed following heat shock and accumulated within the nucleus (Fig. 2A and B). Recently, it was reported that *S. cerevisiae* HSP12 accumulates in the plasma membrane and cell wall when cells enter stationary phase, contributing to cell rigidity [17–19]. In contrast, out data suggested that *S. pombe* Hsp9 accumulates in the nucleus after exposure to heat stress. Therefore, although two HSPs, *S. pombe* Hsp9 and *S. cerevisiae* HSP12, belong to the same small HSP family based on sequence homology, our data suggest that there are differences in the physiological functions of *S. pombe* Hsp9 and *S. cerevisiae* HSP12 in response to cellular stresses.

Interestingly, Δ*hsp9* cells exhibited faster cell growth and short morphology in rich medium (Fig. 2C). The Δ*hsp9* cells did not arrest at the proper time after exposure to heat stress, and then exhibited defective regulation of septum formation and cell death (Fig. 2D). These cells also continued to divide even under stationary phase conditions, and survival was quickly reduced after long-term cultivation under stationary conditions (Fig. 2E and F). These results indicated that the deletion of *hsp9* gene resulted in defective cell cycle arrest under nutrient depletion. In addition, the *hsp9*-overexpressing cells were more elongated and grew slowly (Fig. 2C and E). These phenotypes of *hsp9*-overexpression correspond to that of Δ*hsp9* cells having short length and fast growth in the absence of stress. These results suggest that Hsp9 may be involved in the cell cycle checkpoint that allows progression through the cell-cycle or arrest in response to stresses.

3.4. Hsp9 may play a role in the G2-M checkpoint by modulating the Cdc2 phosphorylation via Cdc25 phosphatase

In order to determine the function of Hsp9 in the cell cycle regulation, we analyzed the timing of nuclear division and the doubling time of Δ*hsp9* cells vs. wild type. The cells were synchronized in the

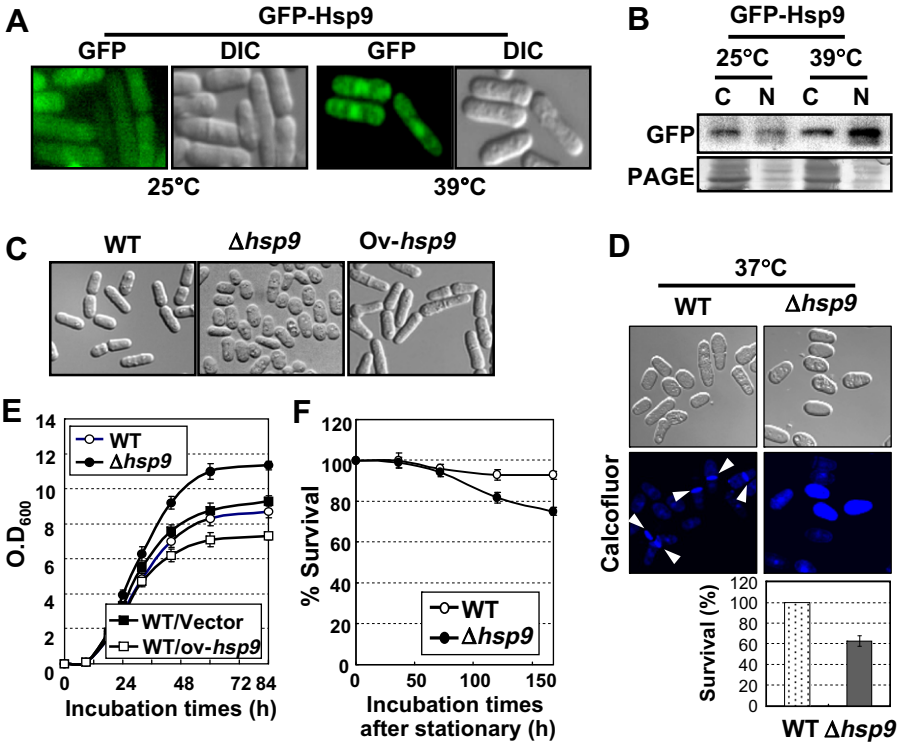

Fig. 2. Hsp9 probably plays a role in cell cycle regulation. (A) *In situ* localization of Hsp9. Cells was examined by fluorescence microscopy after 40 min of heat shock at 42 °C. DIC, differential interference contrast. (B) Hsp9 localization in cell fractions. C, Cytosol; N, Nuclear. Stained SDS–PAGE gel used as a control. (C) The morphology of the Δ*hsp9* mutant and *hsp9*-overexpressing cells at 30 °C. (D) The Δ*hsp9* cells showed a defect in cell cycle checkpoint under heat stress. The wild-type cells and Δ*hsp9* mutant were grown in liquid medium for 24 h at 37 °C. Survival and septum formation were monitored. The septa were stained with calcofluor and are represented by solid white arrows. (E) The growth curves of the Δ*hsp9* mutant and *hsp9*-overexpressing cells in rich media. The cells were initially cultured to the exponential phase (A_{600} = 1.0); the same number of cells (4×10^6 cells mL^{-1}) were transferred to fresh media, and growth was monitored. (F) Survival of Δ*hsp9* cells in stationary phase. The wild-type (WT) and Δ*hsp9* cells were grown to late stationary phase (A_{600} = 8 ∼ 12). The same volume of cultures was sampled at the indicated time points, diluted, and spread onto YES plates. After 3 days incubation, the colonies were counted.

G2 phase by centrifugal elutriation and subjected to fresh culture in minimal media; cell cycle progression was assessed by determining the percentage of cells undergoing nuclear division. Deletion of *hsp9* facilitated nuclear division and shortened generation time (about 120 vs. 140 min) in comparison to G2-synchronized wild-type cells (Fig. 3A). These results suggested that the abbreviated cell cycle in Δ*hsp9* cells is due to early enter to mitosis. Cdc2 kinase activity is negatively regulated by tyrosine-15 phosphorylation in the G2 phase of the cell cycle and enhanced by mitotic dephosphorylation [28,29]. Thus, to verify that *hsp9* deletion results in an early mitotic process, the relative levels of Cdc2-phosphorylation were determined by immunoblot analysis. In wild-type cells, the phosphorylation levels of Cdc2 compared with constant levels of Cdc2 protein declined 45 ∼ 75 min after re-culture, consistent with cells having begun to enter mitosis after 45-min of culture (Fig. 3B). In contrast, Cdc2 dephosphorylation was faster in Δ*hsp9* cells than in wild-type cells for at least 15 min, consistent with facilitated mitosis in these cells (Fig. 3B). In addition, *hsp9* overexpression led to growth retardation and an increased population of G2/M cells that possessed a septum (Fig. 3C and D). These results suggest that *hsp9* may play a role in cell cycle checkpoint at G2-M phase.

In *S. pombe*, many studies have suggested that cell length at division is influenced by the doses of Wee1 and Cdc25 and that timing of mitosis in a normal cell cycle is controlled by Cdc2 phosphorylation [30]. Therefore, in order to clarify the functions of *hsp9* in cell cycle regulation, we assessed the thermo-sensitivity (Fig. 3E) and morphology (Fig. S1) of *ts* mutants, *cdc2-33*, *cdc25-22*, and *wee1* overexpressing *hsp9* using a spot-plate assay. The *hsp9*-overexpression suppressed the *ts* phenotypes of *cdc2-33* and *cdc25-22* mutants, but the *wee1-50* mutant overexpressing *hsp9* showed no difference in cell survival. Since Wee1 inhibits mitosis by phosphorylation of Cdc2 and Cdc25 induces mitosis by dephosphorylation of Cdc2, Hsp9 seems to regulate the phosphorylation status of Cdc2 by modulating the phosphatase activity of Cdc25.

3.5. Hsp9 might control the cell cycle checkpoint by association with 14-3-3 and Cdc25, and by modulating Cdc25 phosphatase activity

To investigate the mechanism of Hsp9 in G2-M checkpoint regulation under stress conditions, we confirmed the status of phosphorylated Cdc2 in Δ*hsp9* cells with or without heat shock because the phosphorylation status of Cdc2 is critical for the G2-M transition. The level of phosphorylated Cdc2 was reduced in Δ*hsp9* mutants and the effect was more severe in heat-shocked Δ*hsp9* mutants, as expected (Fig. 4A). Next, we verified the difference in Cdc25 phosphatase activity between wild-type and Δ*hsp9* by immunoprecipitation with antibody against 14-3-3. The interaction between 14-3-3 and Cdc25 in Δ*hsp9* cells significantly decreased without exerting any effect on the expression of Cdc2 and Cdc25 in both normal and heat-stress conditions, indicating that Hsp9 is likely involved in cell cycle control through regulating the interaction between 14-3-3 and Cdc25 (Fig. 4B). Then, we examined whether Hsp9 directly interacts with 14-3-3 by immunoprecipitation studies using GFP-Hsp9 fusion protein. As shown in Fig. 4C, it was clearly shown that Hsp9 interacts with 14-3-3 and Cdc25 *in vitro*. Many studies in fission yeast have suggested that the two *S. pombe* 14-3-3 proteins, Rad24 and Rad25, appear to suppress the checkpoint defect by inhibiting Cdc25, restoring checkpoint control [31–33]. To confirm the correlation between Hsp9 and 14-3-3 in *S. pombe*, we assessed the UV sensitivity of

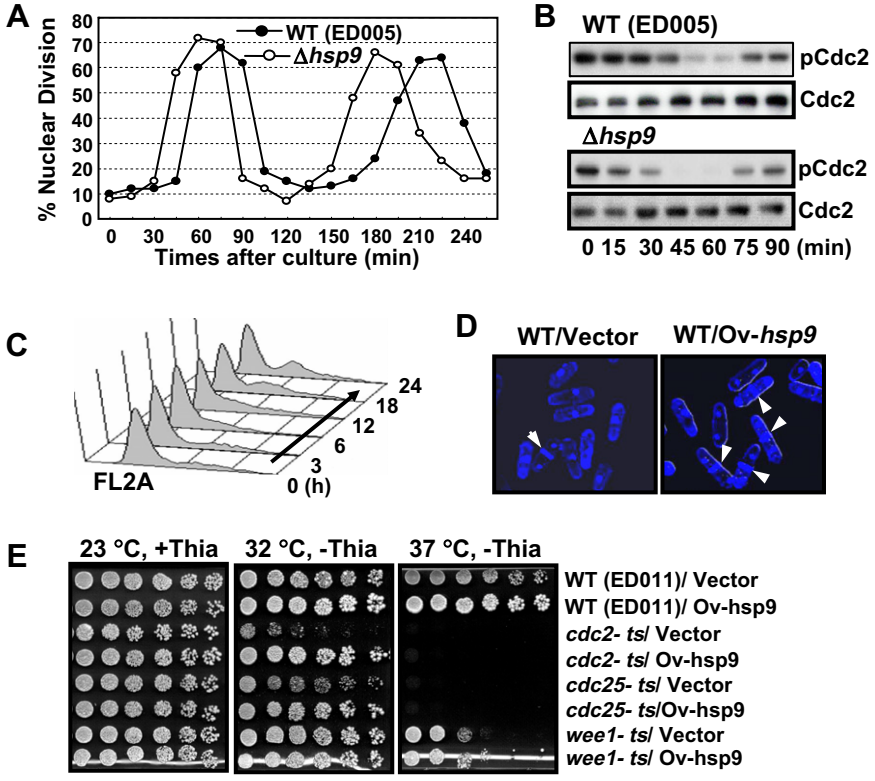

Fig. 3. Hsp9 might play a role in G2-M checkpoint by modulating the Cdc2 phosphorylation. (A) The Δ*hsp9* cells showed early enter to mitosis. Hsp9 may play a role in cell cycle regulation of G2/M phase. Cells were synchronized by centrifugal elutriation by using a JE-5.0 elutriation rotor at 2500 rpm. The synchronized cells resuspended in minimal media, the aliquots were removed at 15-min intervals and counted the cells undergoing nuclear division. (B) The timing of Cdc2 dephosphorylation. (C) Flow cytometry analysis. The *hsp9* gene was induced for the indicated times. (D) The septa of cells overexpressing *hsp9*. The septa were stained with calcofluor after 24 h of *hsp9* induction. (E) Acquisition of thermo-tolerance by *hsp9* overexpression. The wild-type ED665 and mutants *cdc2-33*, *cdc25-2*, and *wee1-50* containing p*nmt1* or p*nmt1-hsp9* were first grown at 23 °C, pre-incubated for 3 h at 23, 33, and 37 °C, and spotted on YES plates. The plates were incubated at 23°C, 33°C, and 37 °C for 48 h.

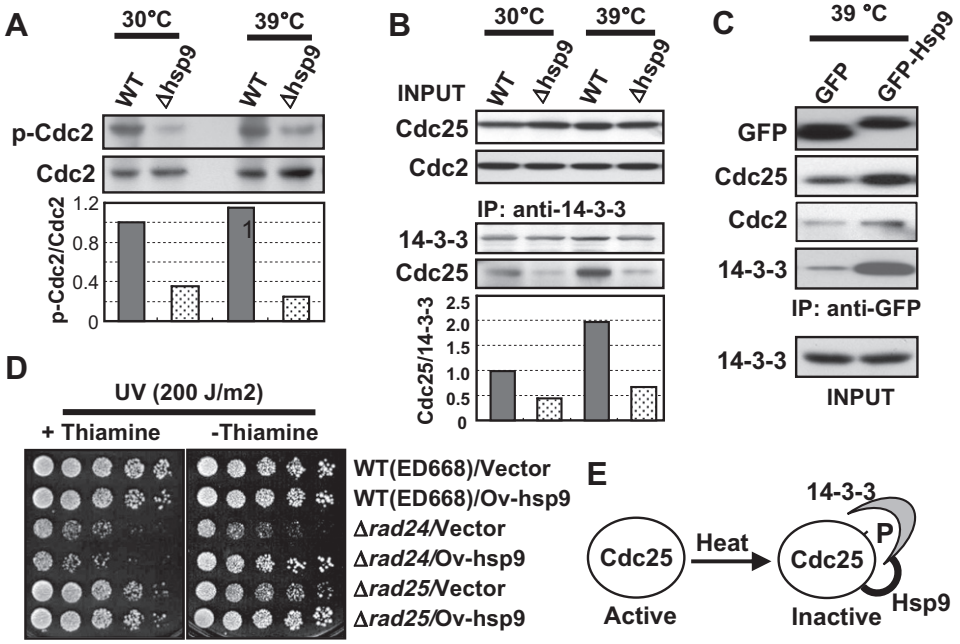

Fig. 4. Hsp9 protein complex with 14-3-3 and Cdc25. (A) The activity of Cdc2 in the Δ*hsp9* mutant under heat-stress condition Whole cell extracts were collected and analyzed by immunoblot analysis. (B and C) Immunoprecipitation using an antibody against 14-3-3 or GFP using 200 μg of cell extracts. Total extract (30 μg) was used as INPUT. (D) The effect of *hsp9* overexpression in 14-3-3 mutant strains, Δ*rad24* and Δ*rad25* in *S. pombe*. After Hsp9 induction for 24 h, cells were spotted and irradiated with 200 J/m^2 of UV-light using a UV crosslinker (XL-1000, Spectronics Co.). After incubation for 48 h, survival was monitored. (E) A predicted model of the interaction of Hsp9 with Cdc25 and 14-3-3 during G2-M checkpoint in *S. pombe*.

Δ*rad24* and Δ*rad25* mutants overexpressing *hsp9* (Fig. 4D). Overexpression of *hsp9* significantly attenuated UV sensitivity of Δ*rad24* and Δ*rad25* mutants. Therefore, Hsp9 seems to enhance 14-3-3 and Cdc25 interaction, which might influence the phosphatase activity of Cdc25, and contributes to cell cycle regulation as expected from its nuclear localization (Fig. 4E).

In conclusion, we found a correlation between *hsp9* deletion or *hsp9* overexpression and stress sensitivity or tolerance, which indicates that Hsp9 might be crucial for protection against unfavorable conditions. In addition, Hsp9 interacts with 14-3-3 and Cdc25 in the nucleus, modulates Cdc25 phosphatase activity, controls the phosphorylation status of Cdc2, and regulates cell cycle progression at the G2-M checkpoint in stressed conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.017.

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