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Tim18, a Component of the Mitochondrial Translocator, Mediates Yeast Cell Death Induced by Arsenic

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Abstract—Evidence is presented that Tim18, a mitochondria translocase, plays a role in the previously described apoptosis induced by arsenite in *Saccharomyces cerevisiae*. *Tim18* deletion mutant exhibited resistance to arsenite. After arsenite treatment, both the wild type and Tim18-deficient cells showed reactive oxygen species (ROS) production. Arsenite induced the higher expression of *tim18* in wild type yeast cells. We found that the *tim18* deletion mutant also exhibited resistance to other apoptotic stresses such as acetic acid, H_2O_2, and hyperosmotic stress. These results suggest that Tim18 is important for yeast cell death induced by arsenic, and it may act downstream of ROS production.

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Apoptosis is an active form of cell death with an important role in development, elimination of damaged cells, and maintenance of tissue homeostasis [1]. Deregulation of apoptosis may cause diseases, such as cancers, immune diseases, and neuronal degenerative diseases [2, 3]. Mitochondria are reported to play a crucial role in regulating mammalian apoptosis [4]. Mitochondria-dependent apoptosis involves the permeabilization of mitochondrial membranes, which appears to mediate the release of apoptogenic factors such as cytochrome *c*, SMAC/DIABLO, Endo G, and AIF [5].

Saccharomyces cerevisiae was found to undergo apoptosis showing characteristic makers such as DNA cleavage and phosphatidyl serine externalization [6], and has been used as a model for apoptotic research [7, 8]. Some researchers provide evidence for the involvement of mitochondria in yeast apoptosis [9, 10]. Ludovico et al. reported that translocation of cytochrome *c* and reduction of mitochondrial membrane potential occur in yeast apoptosis induced by acetic acid, and respiratory deficient strains and cytochrome *c* deleted strains show diminished apoptosis upon acetic acid treatment [9]. A yeast ortholog of mammalian AIF, a mitochondria death protein, has been found to be involved in apoptosis induced by H_2O_2 and chronological aging [10].

Arsenic is a traditional drug that has been widely used for over 2000 years in China [11, 12] and was reported to induce complete remission of acute promyelocytic leukemia with no serious adverse events [13, 14]. There are numerous reports on the proapoptotic effects of arsenic in mammalian cell lines through complex signaling pathways [11, 15]. Recently, we have shown that arsenic induces death in yeast cells, which display the most common apoptotic characteristics such as DNA fragmentation and phosphatidyl serine externalization [16]. It was also shown that mitochondria are involved in this arsenic-induced apoptotic process. A genetic screen for proteins involved in arsenic-induced cell death allowed us to find Tim18, a component of the mitochondrial inner membrane.

MATERIALS AND METHODS

Microorganisms and growth conditions. *Saccharomyces cerevisiae* cells were grown in YPD media containing 1% yeast extract, 2% peptone, and 2% glucose at 30°C. All strains used in this study were in BY4742 (MATa

Abbreviations: CFU) colony forming unit; PTP) permeability transition pore; ROS) reactive oxygen species; TUNEL) terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) background. For treatment with apoptotic inducers, yeast cells were grown to early exponential phase (10^7 cells/ml) and then were harvested and resuspended in treatment medium.

Library screening. The yeast deletion library used is a collection of 4757 homozygous diploid *S. cerevisiae* strains (BY4743: MATa/MATa his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0) in which each strain has a single open reading frame replaced with the KanMX4 module. An aliquot of the pooled yeast library was plated on YPD agar plates supplemented with 3 mM sodium arsenite, a lethal concentration of arsenite to wild-type strain. About 100,000 total colonies were plated. Arsenic-resistant colonies were isolated, and serial dilutions were made and spotted on arsenic plates to confirm the original phenotype. Resistant colonies were chosen, and the corresponding genes were identified by PCR and DNA sequencing analysis.

Cell survival. Yeast culture was adjusted to identical OD_{600} and diluted properly in distilled water, and about 200 cells were spread onto YPD plates. The number of surviving colonies was determined after 2 days of incubation at 30°C.

TUNEL. DNA strand breaks were demonstrated by TUNEL with the In Situ Cell Death Detection Kit (Fluorescein; Roche Diagnostics, Switzerland). Yeast cells were fixed with 3.7% (v/v) formaldehyde as described by Madeo et al. [17] and cell walls were digested with snailase. Cells were washed with PBS, incubated in permeabilization solution (0.1% (v/v) Triton X-100 and 0.1% (w/v) sodium citrate) for 2 min on ice, washed twice with PBS, and incubated with 10 μl TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and FITC-dUTP for 60 min at 37°C. Finally the cells were washed three times with PBS and analyzed under a fluorescence microscope (Zeiss Axioskop, Germany).

Reactive oxygen species (ROS) production. This assay was performed as described previously [17]. Briefly, dihydrorhodamine 123 (1 μl of 2.5 mg/ml stock solution in ethanol) was added to 0.1 ml cell suspension (10^7 cells/ml) in treated medium and incubated at 30°C for 30 min. The production of free intracellular radicals was analyzed using a FACS Calibur (Becton Dickinson, USA) at low flow rate with excitation and emission settings of 488 and 525-550 nm (filter FL1), respectively.

Semi-quantitative RT-PCR. Total RNA was isolated using TRIZOL reagent (Invitrogen, USA) according to manufacture's suggestion. For the reverse transcription PCR, 10 μg total RNA was reverse transcribed using a reverse transcriptase system kit according to the manufacturer's instructions (Promega, USA). PCR were performed with cDNA as template to amplify target gene *tim18* and control gene *ACT1*. PCR products were detected by 2% agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Disruption of *tim18* increases cell resistance to arsenic. Following our observation that arsenic induces mitochondria-involved apoptosis in yeast, we performed a genetic screening for yeast pro-apoptotic genes. A

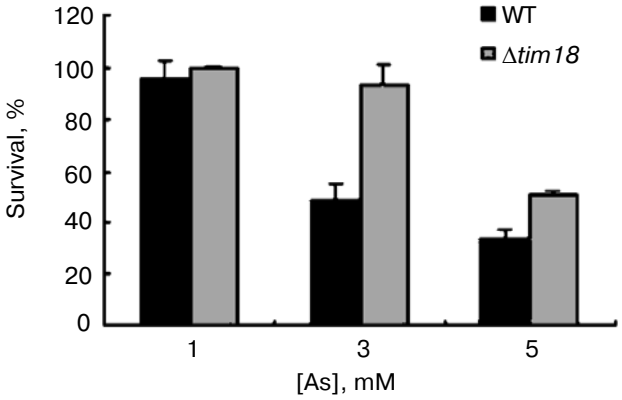

Fig. 1. Relative survival of Δ*tim18* and its isogenic wild type BY4742 in the presence of different concentrations of arsenite.

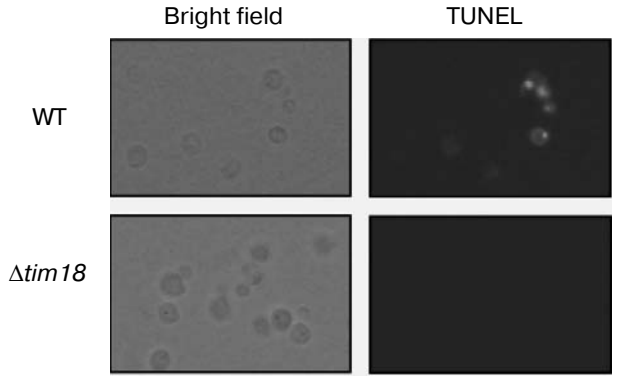

Fig. 2. TUNEL reaction of Δ*tim18* and its isogenic wild type BY4742 treated with 3 mM arsenite.

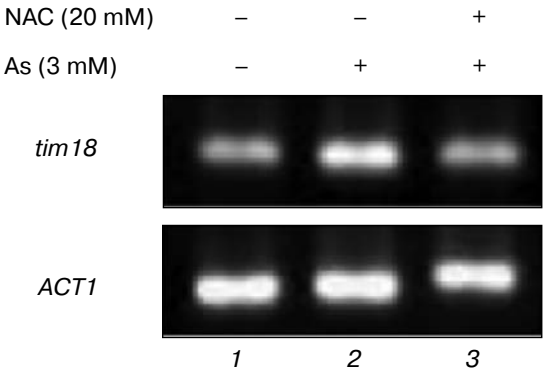

Fig. 3. Expression of *tim18* in yeast cells under different conditions: *1*) untreated; *2*) treated with 3 mM arsenite; *3*) treated with 3 mM arsenite and 20 mM N-acetyl cysteine (NAC).

RESULTS

library comprised of 4757 homozygous deletion strains was plated on YPD agar plates with 3 mM arsenite. Among identified genes, *tim18* attracts our attention for its mitochondrial location [18] and differential expression after arsenic [19]. We analyzed the involvement of Tim18 in arsenic-induced cell death. The *tim18* deletion mutant displayed better survival compared with wild type (Fig. 1). Furthermore, the occurrence of apoptotic marker was strongly reduced compared to wild type (Fig. 2). These results suggest that Tim18 is involved in cell death induced by arsenic.

Arsenic induces *tim18* upregulation. To further understand the functional aspects of Tim18 in mediating arsenic-induced cell death, we use semi-quantitative RT-PCR to examine the expression levels of *tim18* following arsenic treatment in yeast cells. We found that arsenic upregulated the expression of *tim18* in yeast cells (Fig. 3).

Arsenic induces ROS accumulation. ROS production was reported to play an important role in yeast apoptosis [17]. We observed ROS production during arsenic-induced apoptosis in previous work [16]. Interestingly, arsenic induced a similar increase in the levels of ROS in Δ*tim18* mutant strain and wild type cells (Fig. 4), suggesting that Tim18 probably acts downstream of ROS formation. To further investigate the role of ROS in arsenic-induced apoptosis, we tested the effect of antioxidant N-acetyl cysteine (NAC) on this process. We found 20 mM NAC inhibited the lethal effect of arsenic in BY4742 (Fig. 5). Moreover, co-incubation with 20 mM NAC prevented Tim18 overexpression (Fig. 3). These findings supported our hypothesis that Tim18 acts downstream of ROS.

Deletion of *tim18* confers resistance to other apoptotic stimuli. Acetic acid, H_2O_2, and hyperosmotic stress were reported to induce ROS production and apoptosis in yeast [17, 20, 21]. To examine whether Tim18 affects the cell response to apoptotic triggers other than arsenic, cell survival of the strain lacking Tim18 was assessed. The Δ*tim18* mutant strain was found to be more resistant to death induced by acetic acid, H_2O_2, and hyperosmotic stress compared with wild type yeast cells (Fig. 6).

DISCUSSION

In previous work, we found mitochondria were involved in arsenic-induced apoptosis in yeast [16]. In the present study, we identified Tim18, which is located in mitochondria inner membrane, as being possibly involved in this apoptotic process. The mitochondrial inner membrane contains two separate translocons: one required for the translocation of matrix-targeted proteins (Tim23–Tim17 complex) and one for the insertion of polytopic proteins into the mitochondrial inner membrane (Tim54–Tim22 complex). Tim18 protein is part of the Tim54–Tim22 complex, which may function in the assembly and stabilization of the Tim22 complex [18].

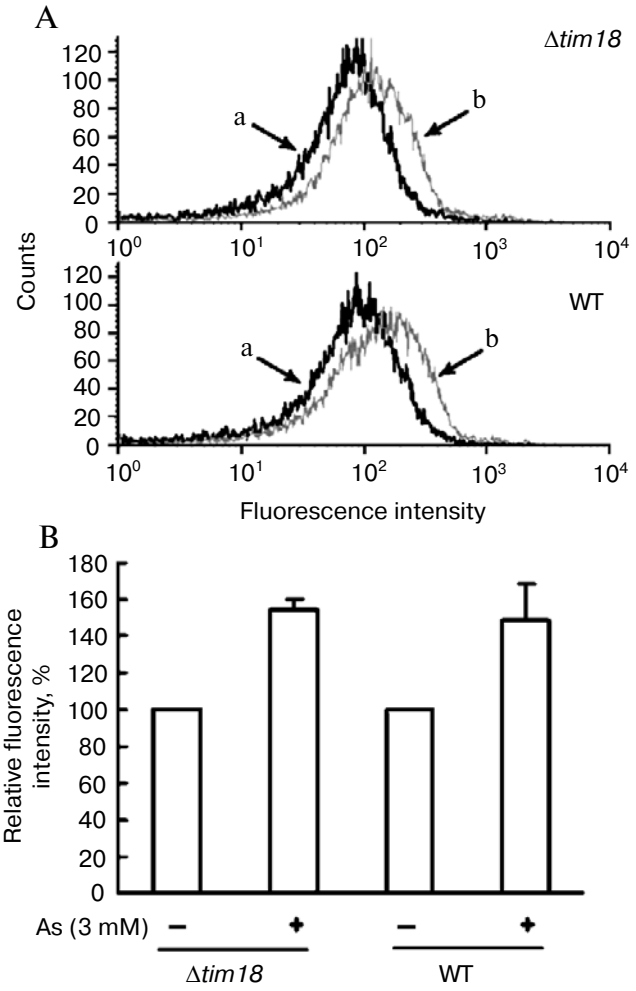

Fig. 4. ROS accumulation in Δ*tim18* and its isogenic wild type BY4742 treated with 3 mM arsenite. A) ROS accumulation in Δ*tim18* and its isogenic wild type BY4742 in the absence (a) or presence of arsenite (b) was measured by flow cytometry. B) The data were normalized against untreated control (100% corresponds to the value of untreated cells).

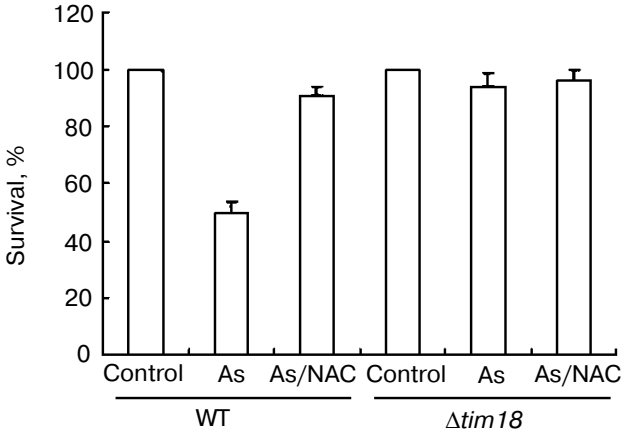

Fig. 5. Cell survival evaluated by CFU (100% corresponds to the number of untreated cells) of Δ*tim18* and its isogenic wild type BY4742 co-incubated with 20 mM NAC.

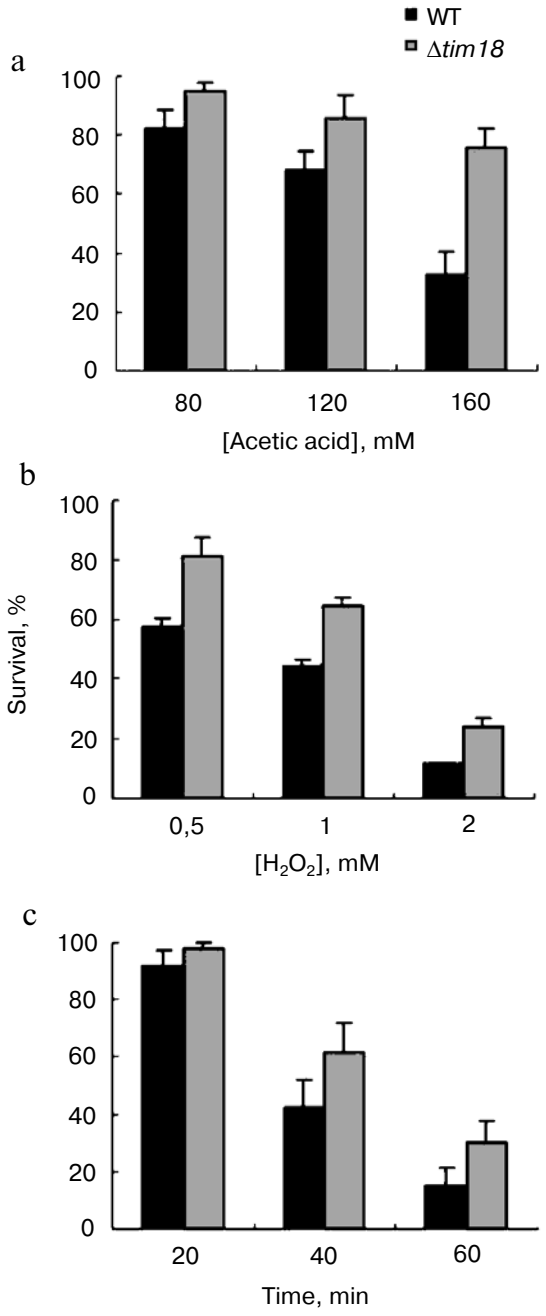

Fig. 6. Cell survival evaluated by CFU (100% corresponds to the number of untreated cells) of Δ*tim18* and its isogenic wild type BY4742 treated by different stimuli: a) 200 min in the presence of acetic acid; b) 200 min in the presence of H_2O_2; c) in presence of 60% (w/w) glucose.

Tim22, the essential core of the mitochondria protein insertion complex, can also form a voltage-activated and signal-gated channel [22]. We found deletion of *tim18* increased the resistance of yeast cells to different apoptotic stimuli including arsenic, acetic acid, H_2O_2, and hyperosmotic stress. These results indicate the participation of Tim18 in yeast apoptosis. We tested the growth of Δ*tim18* and its isogenic wild type BY4742 in liquid YPD and found their growth curves were almost identical (data not shown). Some studies have addressed the role of components of the other mitochondrial translocase in the regulation of the mitochondrial apoptotic pathway. Tim22 is a mitochondrial receptor for the proapoptotic protein Bax. Tim50 regulates the integrity of the outer mitochondrial membrane and cell death [23].

An interesting finding was that arsenic upregulated the expression of *tim18* in yeast cells. This suggests that Tim18 could potentially serve as a biological stress sensor to arsenic, either directly or indirectly. This finding that arsenic upregulated the expression levels of *tim18* is consistent with Haugen et al. who investigated the change in expression profiles after arsenic treatment in genome scale [19].

ROS have been suggested to play a critical role in yeast apoptosis. In mammalian cells, arsenic treatment results in increased intracellular levels of ROS, which lowers mitochondrial membrane potential and leads to cytochrome *c* release and apoptosis [15, 24]. In response to arsenic, Δ*tim18* mutant strain and wild type cells exhibited similar change in the levels of ROS, suggesting that Tim18 probably acts downstream of ROS formation. And this suggestion was supported by the fact that antioxidant NAC prevents *tim18* up-regulation and yeast cell death induced by arsenic.

In summary, we found that a translocase of mitochondrial inner membrane Tim18 plays a role in arsenic-induced cell death. We also suggest that Tim18 probably acts downstream of ROS production during arsenic-triggered apoptosis. Moreover, arsenic induced the increased expression of *tim18* in yeast. Deficiency of Tim18 also affects the yeast response to other apoptotic stress such as acetic acid, H_2O_2, and hyperosmotic stress. Further studies are required to investigate the mechanism of the protein in mitochondria-mediated apoptosis.

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REFERENCES

1. Green, D. R., and Evan, G. I. (2002) *Cancer Cell*, **1**, 19-30.
2. Hickman, J. A. (2002) *Curr. Opin. Genet. Dev.*, **12**, 67-72.
3. Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. (2002) *Cell*, **108**, 153-164.
4. Gottlieb, R. A. (2000) *FEBS Lett.*, **482**, 6-12.
5. Wang, X. (2001) *Genes Dev.*, **15**, 2922-2933.

6. Madeo, F., Frohlich, E., and Frohlich, K. U. (1997) *J. Cell. Biol.*, **139**, 729-734.
7. Madeo, F., Herker, E., Wissing, S., Jungwirth, H., Eisenberg, T., and Frohlich, K. U. (2004) *Curr. Opin. Microbiol.*, **7**, 655-660.
8. Knorre, D. A., Smirnova, E. A., and Severin, F. F. (2005) *Biochemistry (Moscow)*, **70**, 264-266.
9. Ludovico, P., Rodrigues, F., Almeida, A., Silva, M. T., Barrientos, A., and Corte-Real, M. (2002) *Mol. Biol. Cell*, **13**, 2598-2606.
10. Wissing, S., Ludovico, P., Herker, E., Buttner, S., Engelhardt, S. M., Decker, T., Link, A., Proksch, A., Rodrigues, F., Corte-Real, M., Frohlich, K. U., Manns, J., Cande, C., Sigrist, S. J., Kroemer, G., and Madeo, F. (2004) *J. Cell Biol.*, **166**, 969-974.
11. Chen, G. Q., Zhu, J., Shi, X. G., Ni, J. H., Zhong, H. J., Si, G. Y., Jin, X. L., Tang, W., Li, X. S., Xong, S. M., Shen, Z. X., Sun, G. L., Ma, J., Zhang, P., Zhang, T. D., Gazin, C., Naoe, T., Chen, S. J., Wang, Z. Y., and Chen, Z. (1996) *Blood*, **88**, 1052-1061.
12. Aposhian, H. V. (1997) *Annu. Rev. Pharmacol. Toxicol.*, **37**, 397-419.
13. Shen, Z. X., Chen, G. Q., Ni, J. H., Li, X. S., Xiong, S. M., Qiu, Q. Y., Zhu, J., Tang, W., Sun, G. L., Yang, K. Q., Chen, Y., Zhou, L., Fang, Z. W., Wang, Y. T., Ma, J., Zhang, P., Zhang, T. D., Chen, S. J., Chen, Z., and Wang, Z. Y. (1997) *Blood*, **89**, 3354-3360.
14. Soignet, S. L., Maslak, P., Wang, Z. G., Jhanwar, S., Calleja, E., Dardashti, L. J., Corso, D., DeBlasio, A., Gabrilove, J., Scheinberg, D. A., Pandolfi, P. P., and Warrell, R. P., Jr. (1998) *N. Engl. J. Med.*, **339**, 1341-1348.
15. Miller, W. H., Schipper, H. M., Jr., Lee, J. S., Singer, J., and Waxman, S. (2002) *Cancer Res.*, **62**, 3893-3903.
16. Du, L., Yu, Y., Chen, J., Liu, Y., Xia, Y., and Liu, X. (2007) *FEMS Yeast Res.*, in press.
17. Madeo, F., Frohlich, E., Ligr, M., Grey, M., Sigrist, S. J., Wolf, D. H., and Frohlich, K. U. (1999) *J. Cell Biol.*, **145**, 757-767.
18. Kerscher, O., Sepuri, N. B., and Jensen, R. E. (2000) *Mol. Biol. Cell*, **11**, 103-116.
19. Haugen, A. C., Kelly, R., Collins, J. B., Tucker, C. J., Deng, C., Afshari, C. A., Brown, J. M., Ideker, T., and Van, H. B. (2004) *Genome Biol.*, **5**, R95.
20. Ludovico, P., Sousa, M. J., Silva, M. T., Leao, C., and Corte-Real, M. (2001) *Microbiology*, **147**, 2409-2415.
21. Silva, R. D., Sotoca, R., Johansson, B., Ludovico, P., Sansonetty, F., Silva, M. T., Peinado, J. M., and Corte-Real, M. (2005) *Mol. Microbiol.*, **58**, 824-834.
22. Kovermann, P., Truscott, K. N., Guiard, B., Rehling, P., Sepuri, N. B., Muller, H., Jensen, R. E., Wagner, R., and Pfanner, N. (2002) *Mol. Cell*, **9**, 363-373.
23. Guo, Y., Cheong, N., Zhang, Z., de Rose, R., Deng, Y., Farber, S. A., Fernandes-Alnemri, T., and Alnemri, E. S. (2004) *J. Biol. Chem.*, **279**, 24813-24825.
24. Shimizu, S., Matsuoka, Y., Shinohara, Y., Yoneda, Y., and Tsujimoto, Y. (2001) *J. Cell Biol.*, **152**, 237-250.