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SHORT REPORT

Persistent actin depolarization caused by ethanol induces the formation of multiple small cortical septin rings in yeast

Sena Homoto and Shingo Izawa*

ABSTRACT

Short-term exposure to severe ethanol stress has adverse effects on yeast cells. However, limited information is available on the effects of long-term exposure to severe ethanol stress. In this study, we examined the effects of a long-term treatment with a high ethanol concentration [10% (v/v)] on yeast morphology. We found that long-term severe ethanol stress induced the continuous depolarization of the actin cytoskeleton and hypertrophy in yeast cells, accompanied by the aberrant localization of septins, which formed multiple small cortical rings (MSCRs). The formation of MSCRs was also induced by the continuous depolarization of the actin cytoskeleton caused by a treatment with latrunculin-A, an effective inhibitor of actin polymerization. Unlike the formation of conventional septin rings, the formation of MSCRs did not require Cdc42 and its effectors, Gic1, Gic2 and Cla4. These results provide novel insights into the effects of persistent actin depolarization caused by long-term exposure to severe ethanol stress on yeast cytomorphology.

KEY WORDS: Septin, Actin, Cdc42, Ethanol stress, Yeast, Multiple small cortical rings of septin

INTRODUCTION

Saccharomyces cerevisiae produces ethanol through alcoholic fermentation and has a higher tolerance to ethanol than other microorganisms. However, high ethanol concentrations are toxic, even for yeast cells, causing various adverse effects on yeast growth and metabolism. Severe ethanol stress inhibits the activity of amino acid permeases and the glucose transport system (Leão and van Uden, 1982; Alexandre et al., 1998) and increases the fluidity of the plasma membrane and unsaturated fatty acid levels (Daum et al., 1998; You et al., 2003). We also reported that severe ethanol stress [>10% (v/v)] causes the nuclear accumulation of bulk poly(A)$^+$ mRNA and pronounced translation repression, which are accompanied by the formation of processing bodies and stress granules (Takemura et al., 2004; Izawa et al., 2004, 2007; Kato et al., 2011; Yamauchi and Izawa, 2016). These studies mainly reported the effects of short-term ethanol stress on yeast cells.

In contrast, limited information is currently available on the effects of a long-term exposure to severe ethanol stress on yeast cells. During the typical fermentation process of wine, Japanese sake and high-alcohol beers, such as barley wine and strong ales, ethanol concentrations increase to higher than 10% and yeast cells are subjected to high ethanol concentration stress for a relatively long period. Sake brewers have long recognized that yeast cells often exhibit malformation after the completion of sake brewing. Morphological changes in budding yeast are controlled by polarized growth, budding, cytokinesis and septum formation, each of which involves cytoskeletal proteins, such as actin and septins (Bi and Park, 2012; Martin and Arkowitz, 2014; Mishra et al., 2014). The polarity of *S. cerevisiae* is established by the polarized actin cytoskeleton, which is controlled by the small Rho-like GTPase Cdc42, the master regulator of the establishment and maintenance of cell polarity (Park and Bi, 2007; Bi and Park, 2012; Martin and Arkowitz, 2014; Woods et al., 2015; Juanes and Piatti, 2016). The transient depolarization of the actin cytoskeleton is induced in response to various types of stresses, including ethanol (Chowdhury et al., 1992; Lillie and Brown, 1994; Kubota et al., 2004; Uesono et al., 2004).

Cdc42 also controls septin organization through the cell cycle (Caviston et al., 2003; Kozubowski et al., 2005; Gladfelter et al., 2005; Park and Bi, 2007; Okada et al., 2013). Septins are a family of cytoskeletal GTP-binding proteins that are present in all eukaryotes, except higher plants (Pan et al., 2007), and they are required for proper cytokinesis and bud site selection (Hartwell, 1971; Carroll et al., 1998; Douglas et al., 2005; Park and Bi, 2007; McMurray et al., 2011). Therefore, various mutations in septin genes lead to aberrant morphologies in *S. cerevisiae* and *Neurospora crassa* (Gladfelter et al., 2005; Berepiki and Read, 2013).

S. cerevisiae has five mitotic septin proteins (Cdc3, Cdc10, Cdc11, Cdc12 and Shs1) that form hetero-octamers, called rods, in the order of Cdc11–Cdc12–Cdc3–Cdc10–Cdc10–Cdc3–Cdc12–Cdc11, in which Shs1 sometimes replaces Cdc11 (Bertin et al., 2008; Garcia et al., 2011; Bertin and Nogales, 2012). Previous studies have reported that these rods form filaments via self-association and they are maintained throughout many cycles of higher-order polymerization and depolymerization (Frazier et al., 1998; Douglas et al., 2005; Bertin et al., 2010; Garcia et al., 2011; Johnson et al., 2015). During vegetative growth, yeast septin filaments accumulate at the incipient site of bud emergence and then assemble in a ring-like structure at the bud neck, termed the septin ring (Kozubowski et al., 2005; Okada et al., 2013). Concomitant with bud growth, the septin ring expands into a collar or hourglass-shaped tube at the bud neck and then splits into two rings at the onset of cytokinesis (Byers and Goetsch, 1976; Dobbelaere et al., 2003; Dobbelaere and Barral, 2004; McMurray et al., 2011). Septin-based structures act as a scaffold to anchor the regulatory proteins that participate in cell morphogenesis, and also serve as diffusion barriers through their close association with membranes to control molecular trafficking between daughter and mother cells (Kozubowski et al., 2005; Caudron and Barral, 2009; Orlando et al., 2011; McMurray et al., 2011; Wloka and Bi, 2012; Bridges and Gladfelter, 2015; Glomb and Gronemeyer, 2016).

In the present study, we examined the effects of long-term severe ethanol stress [10% (v/v)] on yeast morphology. We found that

Laboratory of Microbial Technology, Graduate School of Science and Technology, Kyoto Institute of Technology, Kyoto 606-8585, Japan.

*Author for correspondence (thioredoxin@kit.ac.jp)

S.I., 0000-0002-4920-2450

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long-term severe ethanol stress induced the continuous depolarization of the actin cytoskeleton and hypertrophy of yeast cells, accompanied by the aberrant localization of septins, which formed multiple small cortical rings (MSCRs). The formation of MSCRs did not require Cdc42 and its effectors, Gic1, Gic2 and Cla4. The formation of MSCRs was also induced by the continuous depolarization of the actin cytoskeleton caused by a treatment with latrunculin-A (LatA), an effective inhibitor of actin polymerization. These results provide novel insights into the effects of long-term high ethanol concentration stress on yeast cytomorphology.

RESULTS AND DISCUSSION

Effects of severe ethanol stress on cell growth and morphology

Kubota et al. (2004) reported that 6% ethanol caused an enlargement in cell sizes by delaying interphase in the cell cycle; therefore, we initially examined changes in cell morphology and size under long-term severe ethanol stress [10% (v/v)]. As shown in Fig. 1A, most non-budding cells were enlarged after the treatment with 10% ethanol stress for 24 h. The average sizes of non-budding cells treated with 10% ethanol for 24 h were 6.46±0.57 and 6.06±0.66 µm (mean±s.d.; long-axis and short axis, respectively) and were larger than those of non-budding cells under non-stressed conditions (5.13±0.34 and 4.44±0.26 µm, respectively). A more prolonged treatment with severe ethanol stress resulted in the appearance of extremely large and elongated cells (Fig. 1A). The ethanol concentration in the medium remained unchanged and was maintained throughout 72 h of cultivation [9.69±0.06% (v/v)]. These results confirmed that, similar to what was seen with 6% ethanol, the long-term treatment with 10% ethanol caused the enlargement of yeast cells.

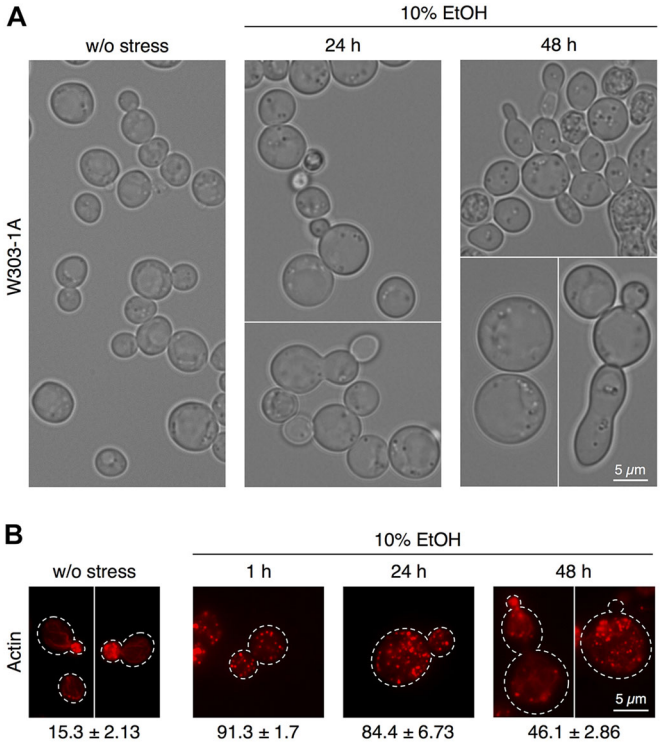

Fig. 1. Effects of severe ethanol stress on cell growth and morphology. (A) Morphological changes in W303-1A under long-term severe ethanol stress (10% ethanol). Pictures of representative cell shapes are indicated. (B) Cells treated with 10% ethanol were stained for F-actin using Rhodamine–phalloidin. The numbers under the panels indicate the percentages of non-polarized cells.

Effects of severe ethanol stress on the actin cytoskeleton

Cytoskeletal organization is crucial for the proper progression of the cell cycle and polarized growth in yeast (Lew, 2000; Michelot and Drubin, 2011). Additionally, 6% ethanol stress was shown to cause transient depolarization of the actin cytoskeleton (for ∼1 h) and a G2 phase delay (Kubota et al., 2004). Therefore, we examined the effects of severe ethanol stress on the actin cytoskeleton. F-actin staining with Rhodamine–phalloidin showed that actin cables disappeared, and actin patches became diffuse in yeast cells cultivated with 10% ethanol stress for 1 h (Fig. 1B). Additionally, this distribution of actin patches was still observed in more than 80% of cells after 24 h of cultivation with 10% ethanol. These results clearly indicate that a long-term exposure to 10% ethanol stress caused a continuous (not transient) depolarization of the actin cytoskeleton. After 48 h of cultivation under 10% ethanol stress, almost half of the cells still showed a depolarized actin cytoskeleton.

Long-term severe ethanol stress induced the formation of MSCRs

Septins are also involved in the proper progression of the cell cycle and polarized growth of yeast cells (Versele and Thorner, 2005; Bi and Park, 2012). Additionally, Cdc42 regulates the organization of septin rings and the actin cytoskeleton. However, the effects of severe ethanol stress on septins were unknown. Because the polarized actin cytoskeleton was severely disturbed by the long-term exposure to 10% ethanol, we examined the organization of septins under severe ethanol stress. After 3 h of cultivation with 10% ethanol, the filamentous structures of Cdc12–GFP (blue arrowheads) were induced and distributed in the cell cortex (misorganized septins) in non-budding cells, whereas septin rings and septin collars around the bud necks were retained in budding cells (Fig. 2A). After 24 h of cultivation, in addition to the diffusion of filamentous structures, aberrantly shaped (white arrowheads) and the disappearance (yellow arrowheads) of septin rings/collars were observed in budding cells. The formation of multiple small rings of Cdc12–GFP (red arrowheads) was concurrently observed in ∼30% of all cells. These small rings formed in the cortical region of yeast cells (Fig. 2B). The formation of multiple small rings was also observed in other strains (YPH250, BY4743 and UT-1) after the treatment with 10% ethanol for 24 h (Fig. 2C).

We then investigated the intracellular localization of other septin components (Cdc3, Cdc10, Cdc11 and Shs1). The GFP-tagged versions of these components also formed multiple small rings and colocalized with Cdc12–mCherry in cells cultivated with 10% ethanol stress for 24 h (Fig. 2D). We additionally observed the formation of multiple small rings via indirect immunofluorescence of Cdc11 (Fig. 2E). These results suggested that the multiple small rings comprise all mitotic septin proteins. Herein, we call them MSCRs. The MSCRs were ∼0.7 µm in diameter.

Although Cdc3, Cdc11 and Cdc12 are essential for cell survival, the Cdc10- and Shs1-deficient mutants (*cdc10*Δ and *shs1*Δ) are viable in standard SD medium (Iwase et al., 2007). Therefore, we investigated whether MSCRs were formed in these mutants under ethanol stress. Since a deficiency of Cdc10 or Shs1 can be compensated for by other septin components in the formation of septin rings/collars (McMurray et al., 2011), these mutants formed septin rings/collars under non-stressed conditions (Fig. 2F). However, no MSCRs were observed in *cdc10*Δ or *shs1*Δ cells after 24 h of cultivation under 10% ethanol, clearly indicating that MSCRs require Shs1 and Cdc10 for their formation. MSCRs appear to be smaller than conventional septin rings/collars, and Shs1 is essential for forming the small ring structures of septin by

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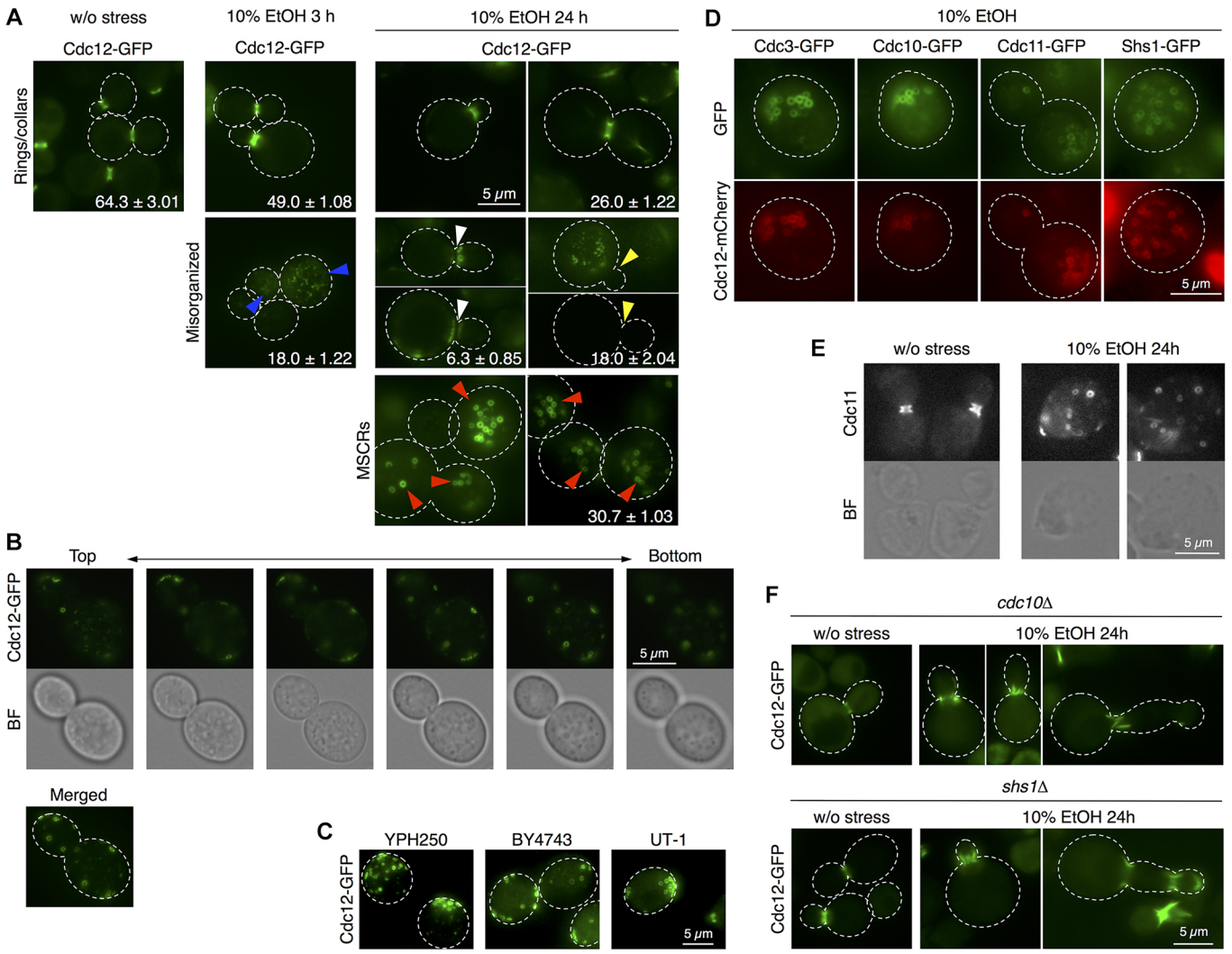

Fig. 2. Formation of MSCRs under severe ethanol stress. The intracellular localization of septin proteins was examined in W303-1A cells under long-term severe ethanol stress. (A) Time-course analysis of Cdc12–GFP under 10% ethanol stress. Arrowheads: blue, filamentous structures; white, aberrantly shaped septin ring; yellow, disappearance of the septin ring; red, multiple small rings. Numbers in the panels indicate the mean±s.d. percentages of each phenotype. (B) *Z*-stack analysis of a cell forming MSCRs. *Z*-stack images were obtained at an interval of 0.7 µm. (C) YPH250, BY4743 and UT-1 cells carrying Cdc12–GFP were treated with 10% ethanol for 24 h. (D) The colocalization of septin proteins was examined using Cdc12–mCherry and other GFP-tagged septin proteins. (E) Indirect immunofluorescence image obtained after staining with anti-Cdc11 antibody. (F) The formation of MSCRs was examined in *cdc10*Δ and *shs1*Δ cells. MSCRs were not detected in these mutants.

promoting the self-assembly of septin filaments (Garcia et al., 2011). In contrast, *cdc10*Δ cells often exhibit wider bud necks with larger septin rings than those in wild-type cells (Frazier et al., 1998; McMurray et al., 2011), and the septin complexes isolated from *cdc10*Δ cells cannot assemble into filaments (Frazier et al., 1998). Therefore, it seems reasonable that *shs1*Δ and *cdc10*Δ cells were unable to induce the formation of MSCRs under severe ethanol stress. Shs1 and Cdc10 are presumably required for the formation of small ring structures such as MSCRs.

MSCRs were quickly disassembled upon the elimination of ethanol

We examined the effects of the elimination of ethanol on MSCRs. After the induction of MSCR formation by cultivation for 24 h with 10% ethanol, cells were transferred into fresh SD medium without ethanol (Fig. 3A). MSCRs completely disappeared within 30–60 min of the elimination of ethanol, and the formation of septin rings was observed around bud necks. A time-lapse analysis demonstrated that the disassembly of MSCRs and formation of septin rings/collars were successively induced after the elimination of ethanol (Fig. 3B). These results indicated that the disassembly of MSCRs and resumption of the formation of septin rings were rapidly induced when ethanol was removed.

Formation of MSCRs caused by severe ethanol stress was independent of Cdc42

The small GTPase Cdc42 and its effectors, such as Gic1 and Gic2 (Gic1/2) and Cla4, are involved in the proper formation of septin rings/collars (Gladfelter et al., 2002; Caviston et al., 2003; Kadota et al., 2004; Versele and Thorner, 2004; Park and Bi, 2007; Iwase et al., 2006; Sadian et al., 2013). Therefore, we investigated whether the formation of MSCRs is induced in a manner that is dependent on Cdc42 and its effectors. *gic1*Δ*gic2*Δ cells and *cla4*Δ cells induced the formation of MSCRs similarly to wild-type cells after 24 h of cultivation in SD medium containing 10% ethanol (Fig. 4A). In the case of the *cdc42-1* mutant, we performed experiments at a

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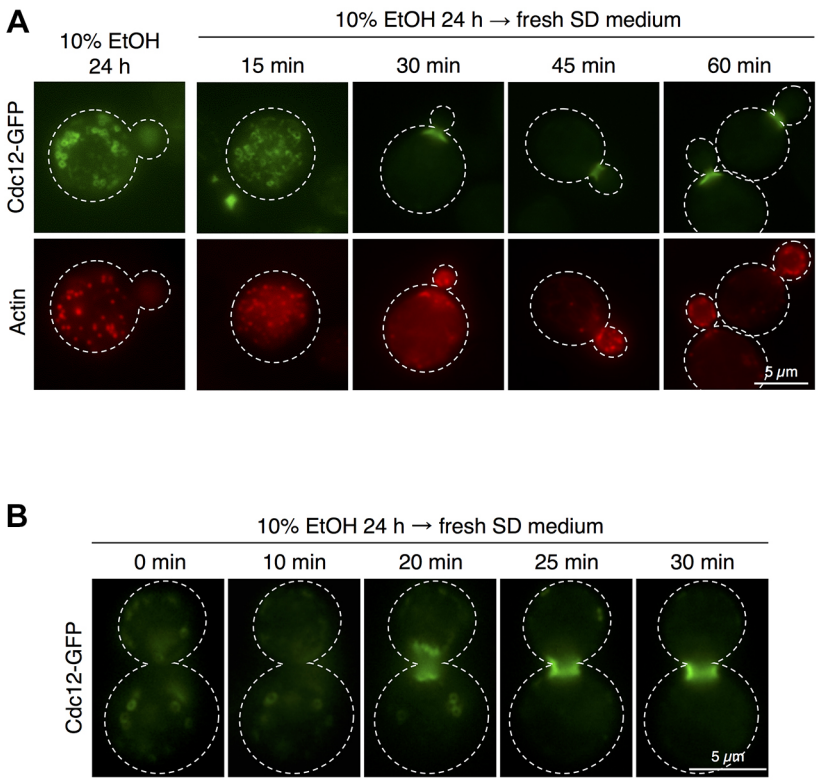

Fig. 3. Elimination of ethanol stress caused the rapid disassembly of MSCRs. After cultivation with 10% ethanol for 24 h, cells were collected and resuspended in fresh SD medium without ethanol. (A) The localization of Cdc12–GFP and the actin cytoskeleton was monitored over time. (B) Pictures of a time-lapse analysis are shown.

permissive temperature (28°C) and restrictive temperature (37°C). *cdc42-1* cells at 28°C had MSCRs under severe ethanol stress (Fig. 4A). *cdc42-1* cells also had MSCRs, accompanied by depolarized actin, after an incubation at 37°C for 5 h, whereas wild-type cells did not (Fig. 4B). These results suggested that, unlike septin rings/collars, the formation of MSCRs under severe ethanol stress does not require the functions of Cdc42, Gic1/2 and Cla4.

Long-term treatment with LatA induced the formation of MSCRs

Since the formation of MSCRs was observed and accompanied by depolarized actin, we speculated that the continuous depolarization of the actin cytoskeleton may be a trigger to induce the formation of MSCRs in yeast cells. Ayscough et al. (1997) reported that the assembly of septin rings was not affected by a treatment with 200 µM LatA, an effective inhibitor of actin polymerization, for a maximum of 4 h. Therefore, we examined the effects of longer treatment times with 200 µM LatA on the formation of MSCRs (Fig. 4C). MSCRs were scarcely observed in cells treated with LatA for 1 h. However, wild-type cells (but not *shs1*Δ or *cdc10*Δ cells) had MSCRs after the treatment with LatA for more than 3 h, and ∼30% of cells had MSCRs after a 5 h treatment with LatA. The LatA-induced formation of MSCRs was verified in other strains using all septin subunits and an anti-Cdc11 antibody (data not shown). These results suggest that the continuous depolarization of the actin cytoskeleton is essential for the formation of MSCRs. Since Cdc42 is involved in the regulation of cell polarity and size, it appears feasible that severe ethanol exerts a continuous inhibitory effect on Cdc42 function that then induces the formation of MSCRs and enlargement of cell sizes (Fig. 1).

Mammalian septin filaments self-assemble and form uniformly curved coils and rings with diameters of 0.6–0.8 µm, which are almost the same size as MSCRs, in an actin-independent manner (Kinoshita et al., 2002). This formation of curved coils and rings was induced not only *in vitro*, but also *in vivo*, in cells in which actin polymerization was blocked by cytochalasin D (Kinoshita et al., 2002). Additionally, the propensity of septin filaments to curl and form rings spontaneously is considered to be well-conserved from yeast to mammals (Kinoshita et al., 2002; Rodal et al., 2005; Garcia et al., 2011). Based on these findings, together with the present results, we propose that MSCRs form via the self-assembly of septin filaments in an actin-independent manner. The spontaneous formation of small rings and coils with mammalian septin filaments takes a relatively long time (Kinoshita et al., 2002); therefore, it seems plausible that the formation of MSCRs took 5–24 h in yeast cells.

Only limited information is currently available on the physiological significance of MSCRs in yeast cells. Although the formation of multiple cortical septin disks, which have a similar size and shape to MSCRs, has been reported in mammalian cells, their physiological functions are also unclear (Sellin et al., 2011, 2014). MSCRs may simply be a meaningless self-assembly structure of septin filaments caused by the disruption of the actin cytoskeleton. However, Ihara et al. (2005) reported that the small cortical septin ring (∼0.6 µm in diameter) in mammalian sperm flagella contributes to the maintenance of the mechanical integrity of spermatozoa. Cortical septins are also known to provide rigidity to the plasma membrane and play a role in the regulation of cell shapes or functions (Tooley et al., 2009; Kim et al., 2010). Based on these findings, the formation of MSCRs may contribute to the integrity of the yeast cell structure under severe ethanol stress. The survival rates of *shs1*Δ and *cdc10*Δ cells, which did not form MSCRs, were lower than those of wild-type cells after cultivation with 10% ethanol for 72 h or 200 µM LatA for 5 h (Fig. 4D). These results might indicate the importance of MSCRs under severe ethanol stress. However, *cdc10*Δ and *shs1*Δ mutants show a decreased rate of vegetative growth at 37°C (Iwase et al., 2007; Sinha et al., 2008; Auesukaree

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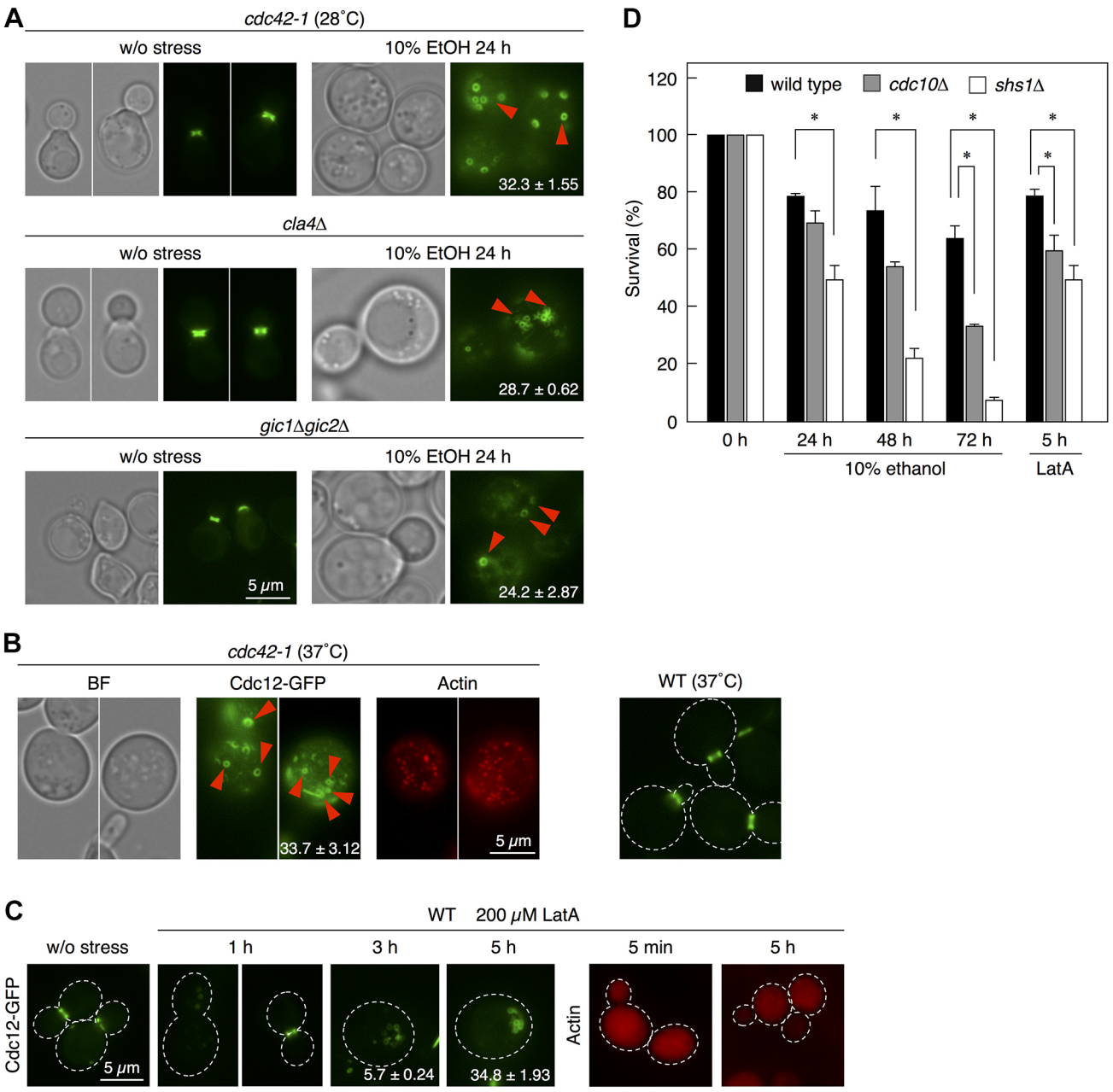

Fig. 4. MSCR formation was induced by the continuous depolarization of the actin cytoskeleton, but independently of Cdc42. (A) Mutant cells were cultured in SD medium with 10% ethanol at 28°C for 24 h. (B) *cdc42-1* cells were incubated in SD medium without ethanol at 37°C (restrictive temperature) for 5 h. (C) Cells were treated with 200 µM LatA for the indicated period. The localization of Cdc12–GFP and the actin cytoskeleton were analyzed. Numbers in the panels indicate the mean±s.d. percentage of cells forming MSCRs. (D) Mean±s.d. survival rates after the treatment with long-term 10% ethanol and LatA were assessed. *P<0.05.

et al., 2009), which did not induce the formation of MSCRs in wild-type cells (data not shown). Therefore, we cannot exclude the possibility that the absence of MSCRs in these mutants is not related to their hypersensitivity to ethanol and LatA. We are planning further careful and detailed studies to clarify the physiological functions of MSCRs.

MATERIALS AND METHODS

Strains and medium

Saccharomyces cerevisiae strain W303-1A (*MAT***a** *his3-11*, *15 leu2-3*, *112 trp1-1 ade2-1 ura3-1 can1-100*) and its isogenic mutants, *cla4*Δ::*CgHIS3*, *cdc10*Δ::*LEU2*, *shs1*Δ::*HIS3*, *cdc42-1* and *gic1*Δ*gic2*Δ, were used in the present study. The mutants *cla4*Δ::*CgHIS3*, *cdcd42-1*, *cdc10*Δ::*LEU2* and *shs1*Δ::*HIS3* (Iwase and Toh-e, 2004; Iwase et al., 2006, 2007) were provided by the National BioResource Project [NBRP; Yeast Genetic Resource Center (YGRC)], Japan. To construct *gic1*Δ*gic2*Δ cells, a DNA fragment (2.5 kb) encoding the *gic1*Δ::*kanMX* region was amplified using genomic DNA from the *gic1*Δ mutant (Open Biosystems Inc., Huntsville, AL) as a template, and the primers 5′-TATGCAGCTGTGTGTCTAGAG-GAGTAAAAC-3′ and 5′-CAACAGTGGAAGAAAAAAACTCGAGGG-TAG-3′. Another DNA fragment (2.5 kb) encoding the *gic2*Δ::*HIS3* region was amplified using the genomic DNA of the *gic2*Δ mutant (Iwase et al., 2006), and the primers 5′-GAAACTTAAAGGATCCCCATTGTCTCAA-A-3′ and 5′-CATCTGAGGTACCTTTACGGTCAATCGTTC-3′. These amplicons were introduced into W303-1A to construct *gic1*Δ*gic2*Δ (*MAT***a**

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his3-11, *15 leu2-3*, *112 trp1-1 ade2-1 ura3-1 can1-100 gic1*Δ::*kanMX gic2*Δ::*HIS3*). YPH250 (*MAT***a** *trp1-1 his3-200 lys2-801 leu2-1 ade2-101 ura3-52*) (Yeast Genetic Stock Center, University of California at Berkeley, CA), BY4743 (*MAT***a**/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0*) (Open Biosystems Inc.), and the sake yeast UT-1 (*MAT***a**/α *ura3/ura3 trp1/trp1*) (Kitamoto et al., 1990) were used to verify the formation of MSCRs. Cells were cultured in 50 ml of SD medium (2% glucose, 0.67% yeast nitrogen base without amino acids, pH 5.3) with appropriate supplements of amino acids and bases at 28°C with reciprocal shaking (120 rpm) in Erlenmeyer flasks (200 ml). Exponentially growing cells were harvested at an optical density at 600 nm (OD_{600})=1.0, transferred into fresh SD medium containing 10% ethanol (v/v), and cultured further. The initial optical density was adjusted to OD_{600}=0.3. To prevent the evaporation of ethanol, flasks were sealed with aluminium foil and Parafilm. Ethanol concentrations in the medium were measured by performing gas chromatography (AL-2; Riken Keiki Co., Tokyo, Japan).

Plasmids

pRS313-CDC12-mCherry was constructed by cloning *CDC12*, containing its promoter and coding region and the mCherry gene into the *Sal*I/*Bam*HI and *Bam*HI/*Xba*I sites of pRS313 (Sikorski and Hieter, 1989), respectively. *CDC12* was amplified using the primers 5′-TAGCTTGAACGGCATTG-TCGACTTTGAACC-3′ and 5′-CAAAGAGGAAGACATTAATTAATG-GATCCTTTAAATGGG-3′, using genomic DNA from W303-1A as the template. The mCherry gene was amplified using the primers 5′-AAGCT-TGCATGCCTGCAGGTCGACTCTAGA-3′ and 5′-TAATGGTAGCGA-CCGGCGCTCAGTTGGAAT-3′, and pmCherry (Clontech, Mountain View, CA) as the template.

pGF316-CDC3-GFP, pGF316-CDC10-GFP, pGF316-CDC11-GFP, pGF316-CDC12-GFP and pGF316-SHS1-GFP (Iwase and Toh-e, 2001) were provided by the NBRP (YGRC), Japan.

Microscopy analysis

A fluorescent microscopic analysis was performed using a Leica AF6500 fluorescence microscopic system (Leica Microsystems Vertrieb GmbH, Wetzlar, Germany). The percentages of cells with misorganized septins or MSCRs were calculated by monitoring 200 cells under each condition, and experiments were repeated three times (a total of 600 cells were examined). Actin patches and cables were stained using the method of Nomura and Inoue (2015). The percentages of non-polarized cells were calculated by monitoring 100 cells under each condition, and experiments were repeated three times (300 cells in total were examined). In the indirect immunofluorescence of Cdc11, cells were fixed with 4% paraformaldehyde, converted to spheroplasts with Zymolyase 20T (Seikagaku Biobusiness, Tokyo, Japan), permeabilized with 0.5% NP-40, and attached to poly L-lysine-coated coverslips as described by Niu et al. (2011). Samples were incubated with an anti-Cdc11 antibody (sc-166271, lot F2012, Santa Cruz Biotechnology, Santa Cruz, CA) as the primary antibody at a 1:100 dilution and anti-mouse IgGκ-BP-CFL 488 as the secondary antibody (SC-516176, Lot C1618, Santa Cruz Biotechnology) at a 1:100 dilution.

Measurement of survival rates and cell sizes

To assess the survival rate, dead cells were stained with 0.02% Methylene Blue solution in 50 mM potassium phosphate buffer, pH 6.8 (Nagai, 1963). The average sizes of non-budding cells were measured using ImageJ software (http://imagej.nih.gov/ij/). A total of 100 cells in each condition were examined, and the experiments were repeated three times (a total of 300 cells were examined).

Chemicals

LatA was purchased from Wako Pure Chemicals (125-04363, Osaka, Japan). All other chemical reagents were also purchased from Wako Pure Chemicals.

Statistical analysis

Data are presented as means±s.d. The significance of differences was assessed using the paired *t*-test. Compared results were considered to be significantly different when the $P<0.05$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Formal analysis: S.H.; Investigation: S.H., S.I.; Writing - original draft: S.H., S.I.; Writing - review & editing: S.I.; Project administration: S.I.; Funding acquisition: S.I.

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