Temperature-sensitive defects of the GSP1gene, yeast Ran homologue, activate the Tel1-dependent pathway

Temperature-sensitive defects of the *GSP1* gene, yeast Ran homologue, activate the Tel1-dependent pathway

Naoyuki Hayashi[1, 2]*, Seishi Murakami[2], Susumu Tsurusaki[3], Zen-ichiro Ngaura[3], Masaya Oki[3]#, Hideo Nishitani[3], Masahiko Kobayashi[1], Hiroko Shimizu[1], Kenichi Yamamoto[1] and Takeharu Nishimoto[3]

1:Department of Molecular Pathology, 2: Department of Molecular Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-0934 Japan, 3: Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582 Japan

*: Corresponding author

e-mail: naoyuki@kenroku.kanazawa-u.ac.jp

Present address:

[3]#, Department of Applied Chemistry & Biotechnology, Faculty of Engineering, University of Fukui, 3-9-1 Bunkyo, Fukui 910-8507, Japan

Abstract

Ran GTPase is involved in many cellular processes. It functioned in nuclear-cytosolic transport and centrosome formation. Ran also localizes to chromatin as RCC1 does, its guanine nucleotide exchange factor, but Ran's function on chromatin is not known. We found that *gsp1*, a temperature-sensitive mutant of *GSP1*, a *Saccharomyces cerevisiae* Ran homologue, suppressed the hydroxyurea (HU) and ultra violet (UV) sensitivities of the *mec1* mutant. In UV-irradiated *mec1 gsp1* cells, Rad53 was phospholyrated despite the lack of Mec1. This suppression depended on the *TEL1* gene, given that the triple mutant, *mec1 gsp1 tel1*, was unable to grow. The *gsp1* mutations also suppressed the HU sensitivity of the *rad9* mutant in a Tel1-dependent manner, but not the HU sensitivity of the *rad53* mutant. These results indicated that Rad53 was activated by the Tel1 pathway in *mec1 gsp1* cells, suggesting that Gsp1 helps regulate the role switching the ATM family kinases Mec1 and Tel1.

Keywords: Ran; Gsp1; checkpoint; ATM kinase; Mec1; Tel1; Rad53; yeast; replication; signal transduction

Introduction

The nuclear small GTPase Ran functions in various cellular spatial processes, as including the nucleocytoplasmic transport of macromolecules, microtubule formation and nuclear membrane assembly [1, 2, 3, 4]. Ran is a small G protein that is functionally and structurally conserved from yeast to mammals [1]. RanGEF (Ran-GDP/GTP exchange factor) and RanGAP (RanGTPase activating protein) are auxiliary factors of Ran. RanGEF, called RCC1 in mammalian cells [5], is localized to the chromatin [6], while RanGAP is mainly localized to the cytoplasm [7]. Therefore, there is a gradient of Ran-GTP concentration from the nucleus to the cytoplasm that determines the destination of protein transport [4]. Nuclear proteins synthesized in the cytoplasm are imported into the nucleus depending on their nuclear localization signal (NLS). Within the nucleus, however, it is unknown how the imported proteins are delivered to their functional targets. One current model suggests that protein localization within the nucleus occurs by random movement [8]. However, this model cannot fully explain the rapidity with which ATR or ATM is delivered to damaged chromosomal DNA sites when the cells are exposed to genotoxic stresses [9]. Therefore, some other mechanism for regulating the movement of nuclear proteins is most likely at work.

One factor that might regulate nuclear protein movement is Ran, since RanGEF, RCC1, is localized to the chromatin, causing a high concentration of Ran-GTP in the nucleus. RCC1 may sense the status of the chromosome, for example, DNA damage, replication or transcription, and transfer these signals to effecter proteins through Ran-GTP [10], thereby eliciting coupled cellular spatial and temporal events. The coupling of spatial and temporal events is thought to be essential for cell proliferation [11]. In agreement with this idea, the loss of RCC1 causes the premature initiation of mitosis in tsBN2 cells derived from the

hamster BHK21 cell line, arrested with hydroxyurea (HU) [12]. In this context, we focused on whether the Ran-mediated processes are related to DNA replication, which is a major timed event in cell proliferation.

To address this possibility, a series of temperature-sensitive mutants of Gsp1, a *Saccharomyces cerevisiae* Ran homologue [13], coupled with a *mec1* mutant, which lacks one of the ATM family kinases, were examined for their sensitivity to HU. We found that the *mec1 gsp1* mutants showed an HU-resistant phenotype in an allele-specific manner. We also found that the viability and HU-resistant phenotype in *mec1 gsp1* cells was dependent on *TEL1*, which encodes the other yeast ATM family kinase. These results indicated that the yeast Ran homologue, Gsp1, is involved in controlling the ATM family kinases.

Materials and methods

Strains and plasmids.

The *S. cerevisiae* strains and plasmids used in this experiment are listed in Table 1. The gene disruption was carried out as previously described [13].

Gene Disruption.

tel1: A DNA fragment between the 6389th and the 6829th bp of the *TEL1* ORF and one containing the 3'-untranslated region of the *TEL1* ORF were amplified, respectively, by the following two sets of primers: 5'-CAT TGT CAA TTG GGC CCT AAA TGT CGA AG-3' and 5'-CCC AGG GCA AAA GGA TCC AGC TCG GTA TTG-3; and 5'-GCT ATG CGG ATC CTT TAT ACT TC-3' and 5'-GAG CCA AAT CCG CGG AGT GAG TC-3'. The primers were designed to introduce, respectively, the restriction enzyme sites *Apa*I, *Bam*HI, *Bam*HI, and *Sac*I at the ends. The two fragments were ligated at their *Bam*HI site, and then inserted into the *Apa*I-*Sac*I sites of pBluescript II SK(+). A *Bam*HI fragment containing *loxP-kanMX-loxP* derived psh47 [14] was inserted into the *Bam*HI site of the resulting plasmid. To disrupt the *TEL1* gene, the *Apa*I-*Sac*I fragment containing *tel1::loxP-kanMX-loxP* of the constructed plasmid was introduced into the yeast strains.

rad50: A 1.5-kb PCR fragment containing a part of the *RAD50* ORF was amplified using a primer pair 5'-TGT CAC CAA GAA GAC AGC CT-3' and 5'-AGT CTT ATA GGA GAG CTC CG-3', and then digested with *Xba*I. The fragment was then cloned into the *Xba*I site on pUC29 [15]. A 2.2-kb *Bgl*II fragment containing the *ADE2* of pASZ11 [16] was inserted into the *Bgl*II site on the resulting plasmid. The constructed plasmid was digested with *Xba*I, and a fragment containing *rad50::ADE2* was introduced into the yeast strain.

Cell growth and cell cycle assays.

Cells were cultivated until $OD_{660\ nm}$ = 1.0 and were sequentially diluted 10-fold. Five microliters of the serial dilutions was spotted on synthetic medium containing 1 mg/ml of 5-fluoro orotic acid (5FOA), 50 mM hydroxyurea (HU), or no additions. The cells were incubated at 26ºC for 3 days.

To examine cell cycle in yeast mutants, we used FACScan analysis. Yeast cells were grown in synthetic medium at 26ºC. HU (final concentration 100 mM) was added into the culture of exponentially growing cells. The cells were harvested, washed with water at various times. For DNA flow cytometry, the cells were fixed in 70% ethanol and then moderately sonicated. Following RNase treatment (1 mg/ml) at 50 ºC for 1 h, the cells were stained with 10 μg/ml propidium iodide in Tris-HCl (pH 7.5), and analyzed on a Becton-Dickinson FACSCalibur [17]. For each experiment, we analyzed approximately 20000 cells.

Immunoblotting Analysis.

Yeast cells were cultivated to the early log phase ($OD_{660\ nm}$=1.0) in 20 ml of synthetic medium at 26 ºC, and 10-ml aliquot of these cultures were transferred to 10-cm dishes. These cultures were irradiated with Funakoshi-UV-linker (Funakoshi, Tokyo, Japan), and then incubated in a dark box at 26 ºC for 1 h. These UV-treated cultures and untreated controls were then disrupted with NaOH and 2-merkaptethanol as described previously [18]. The resulting cell extracts were subjected to SDS-PAGE (7%) and transferred onto nitrocellulose membranes (Pall Corporation, FL, USA). The membranes were stained with antibodies against myc-tag (Sigma-Aldrich Co, MO, USA).

Sensitivities to DNA damage.

Exponentially growing cultures of the yeast cells were diluted and adjusted to $OD_{660\ nm}$ = 1.0. These adjusted cultures were serially diluted 5-fold, and 5 μl of each aliquot was spotted onto a YPDA plate. To assess ultraviolet (UV) sensitivity, the cells spotted on the YPDA plates were irradiated using a Funakoshi-UV-linker (Funakoshi, Tokyo, Japan). For the UV-radiation survival curve, aliquots of appropriately diluted yeast cells were plated and irradiated with various doses of UV. The plates were then incubated at 26°C for 2 days.

For the MMS survival curve, exponentially growing cultures were diluted and adjusted to $OD_{660\ nm}$ = 0.1. After the addition of MMS to a 0.05% final concentration, the cultures were incubated and samples were harvested at 10-min intervals. The harvested cells were resuspended into the same volume of 10% sodium thiosulfate to inactivate the MMS, and tested for viability. Viabilities were estimated by colony counting of aliquots of appropriately diluted yeast cells on the YPDA plates. The initial viabilities of the original culture before exposure of MMS were determined as described above.

Results and discussion

HU sensitivity in *mec1* was suppressed by *gsp1* mutations.

A temperature sensitive mutant of RCC1, the guanine nucleotide exchange factor for Ran GTPase, in a hamster cell line showed G1 arrest in asynchronous culture due to failure of replication initiation, and in synchronous culture the cells begin mitosis with premature chromosomal condensation in the S phase at the restricted temperature [6]. Similar observations of altered cell cycle control in the S phase have been found in budding and fission yeasts [19, 20, 21]. These findings may suggest that Ran GTPase is involved in checkpoint control in the S phase. To study this possibility, we introduced *gsp1* mutations into *mec1*-deficient budding yeast, which lack one of the ATM kinases, which regulate the replication checkpoint. We genetically introduced the *mec1* disruption carried by yeast derived from strain YEF578a (Table 1; kindly provided by Dr. Shirahige) into 6 *gsp1* mutants. HU, which inhibits the biosynthesis of deoxynucleotides, causes replication to stall. HU-treated cells showed an arrested cell cycle in the early S phase and activate ribonucleotide reductases (RNR) to overcome the inhibition by HU. Surprisingly, the HU-sensitive phenotype caused by the *mec1* mutation was suppressed by the *gsp1* mutations, except for the *gsp1-1268* allele (Fig. 1A). Therefore, *gsp1-479* and *-1757* single-mutation alleles were chosen for further study. From the FACS analysis, we found that the two double mutants, *mec1 gsp1-479* and *mec1 gsp1-1757*, continuously proliferated without cell-cycle arrest in the presence of 100 mM HU, although arrest in the early S phase was observed in wild-type cells 4 hours after HU addition (Fig. 1B). These results indicated that the *gsp1* mutations rescued replication deficiency in the *mec1* mutants, but did not rescue the deficiency in cell-cycle arrest.

To confirm the *gsp1*-mediated suppression of the *mec1* phenotypes, we next examined the sensitivity of the double mutant, *mec1 gsp1,* to UV-irradiation and treatment with the genotoxic alkylation agent MMS. Mid log-phase cultures were spotted on YPDA medium and then exposed to the indicated dose of UV irradiation. After UV-irradiation, the cells were incubated in a dark box at 26°C, the permissive temperature for the *gsp1* mutations. As previously reported [22], the *mec1* cells ewre highly sensitive to UV (Fig. 2 Left). In contrast, the *mec1 gsp1-479* and *mec1 gsp1-1757* strains were resistant to UV, to almost the same as the wild-type strain. Both *gsp1-479* and *gsp1-1757* only partially suppressed the sensitivity of the *mec1* cells to MMS (Fig. 2 Right). This partial suppression of MMS sensitivity may suggest that *gsp1* mutations in *mec1* cells overcame the replication defects caused by HU or UV, but could not overcome severe DNA damage, such as the double strand breakage caused by the genotoxic alkylation agent.

Tel1-pathway required for the suppression of the mec1 *phenotype*

We next examined the phosphorylation of Rad53, which is phosphorylated by Mec1 in response to UV treatment (Fig. 3). The mobility of myc-tagged Rad53 on gels was retarded in *mec1 gsp1-479* and *mec1 gsp1-1757* cells as in wild-type cells, indicating that Rad53 was phosphorylated under UV treatment in the absence of Mec1 in the *gsp1* mutants. Two pathways, the Mec1- and the Tel1-dependent pathways, which have some redundancy, are reported to function in replication and DNA-damage checkpoint control [23, 24, 25]. Two ATM family kinases, Mec1 and Tel1, are known to be signal transducers in these pathways. The *mec1* cells lose abilities of checkpoint response and replication progression in spite of the presence of the *TEL1* gene, which has the overlapping functions. Therefore, additional gene disruptions in *mec1 gsp1* were carried out in the presence of a plasmid pRK900, containing

the *MEC1* and *URA3* genes, and then pRK900 was removed by 5FOA treatment for investigation of a possibility that suppression to the *mec1* phenotype by the *gsp1* mutants depends on Tel1 function. None of three triple mutants, *rad50 mec1 gsp1-479, tel1 mec1 gsp1-479* or *rad50 mec1 gsp1-1757,* proliferated in the absence of wild-type *MEC1* on pRK900 (Fig. 4A). One triple mutant, *tel1 mec1 gsp1-1757,* grew slowly in the absence of pRK900, but was sensitive to HU. These observations suggest that Tel1 and probably Rad50/Mre11/Xrs2 complex are crucial for the suppression of the *mec1* phenotype by the *gsp1* mutants.

Rad53 is a common downstream factor of Mec1 and Tel1. It is phosphorylated by either Mec1 or Tel1 in response to replication stall and/or DNA damage [23]. As expected, Rad53 was essential for the *mec1 gsp1* strains to survive in the presence of HU, since the *rad53 gsp1-479* and *rad53 gsp1-1757* strains were sensitive to it (Fig. 4B). In the checkpoint response, an interaction between Rad53 and Rad9 is required for the phosphorylation of Rad53 by Mec1 [26]. Thus, to show that Rad9 as well as was important in the *mec1 gsp1* strains, the double mutants *rad9 gsp1-479* and *rad9 gsp1-1757* were constructed. Both double mutants grew better than *rad9* cell in the presence of 50 mM HU (Fig. 4B). On the other hands, *tel1 rad9 gsp1-1757* cells were quite sensitive to HU, but the *mec1 rad9 gsp1-1757* cells were resistant to it. These observations indicated that the Tel1 function in *gsp1* mutant rescued the Mec1/Rad9 pathway to Rad53, and suggested that Rad53 has a critical role in the Tel1 signal pathway in *gsp1* mutants. In conclusion, *mec1* cells were sensitive to replication stall and DNA damage in spite of presence of an intact *TEL1* gene, but Tel1 took the place of Mec1 in *mec1* cells when *gsp1* was mutated. Therefore, the nuclear G protein, Ran, may function to regulate the role of the ATM family kinases.

The Ran GTPase cycle is candidate for roles in nuclear events related to DNA damage

and replication. We can imagine from the current Ran cycle model that a defect in Ran may reduce the control of protein movement that is important for the control of DNA damage and of the replication checkpoint. This idea is also consistent with our recent finding that a defect in RanGAP causes a centromeric gene-silencing defect in *Schizosaccharomyces pombe* [27].

Acknowledgments

We thank Drs. Harashima, Hishida (Osaka University, Japan), Shirahige (Tokyo Institute of Technology, Japan), Tsukamoto, and Ogawa (Iwate Nurse College, Japan) for providing us with yeast strains. This work was supported by Grants-in-Aid for Specially Promoted Research from the Ministry of Education, Science, Sports, and Culture of Japan.

References

[1] M. Dasso, The Ran GTPase: theme and variations. Curr. Biol. 12 (2002) R502-R508.

[2] J. A. Kahana, D. W. Cleveland, Beyond nuclear transport. Ran-GTP as a determinant of spindle assembly. J. Cell Biol. 146 (1999) 1205-1210.

[3] I. Mattaj, Sorting out the nuclear envelope from the endoplasmic reticulum. Nat. Rev. Mol. Cell Biol. 5 (2004) 65-69.

[4] K. Weis, Regulating access to the genome: nucleocytoplasmic transport through the cell cycle. Cell 112 (2003) 441-451.

[5] R. Kai, M. Ohtsubo, M. Sekiguchi, T. Nishimoto, Molecular cloning of a human gene that regulates chromosome condensation and is essential for cell proliferation. Mol. Cell. Biol. 6 (1986) 2027-2032.

[6] M. Ohtsubo, H. Okazaki, T. Nishimoto, The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA. J. Cell Biol. 109 (1989) 1389-1397.

[7] W. Feng, A.L. Benko, J. H. Lee, D. R. Stanford, A. K. Hopper, Antagonistic effects of NES and NLS motifs determine S. cerevisiae Rna1p subcellular distribution. J. Cell Sci. 112 (1999) 339-347.

[8] T. Pederson, Protein mobility within the nucleus-what are the right moves? Cell 104 (2001) 635-638.

[9] S. M. Barr, C. G. Leung, E. E. Chang, K. A. Cimprich, ATR kinase activity regulates the intranuclear translocation of ATR and RPA following ionizing radiation. Curr. Biol. 13 (2003) 1047-1051.

[10] T. Nishimoto, A new role of Ran GTPase. Biochem. Biophys. Res. Comm. 262 (1999) 571-574.

[11] J. Pines, Four-dimensional control of the cell cycle. Nat. Cell Biol. 1 (1999) E73-E79.

[12] H. Nishitani, M. Ohtsubo, K. Yamashita, H. Iida, J. Pines, H. Yasuda, Y. Shibata, T. Hunter, T. Nishimoto, Loss of RCC1, a nuclear DNA-binding protein, uncouples the completion of DNA replication from the activation of cdc2 protein kinase and mitosis. EMBO J. 10 (1991) 1555-1564.

[13] M. Oki, E. Noguchi, N. Hayashi, T. Nishimoto, Nuclear protein import, but not mRNA export, is defective in all of the temperature-sensitive mutants of the *Saccharomyces cerevisiae* Ran homologue, Gsp1-GTPase. Mol. Gen. Genet. 257 (1998) 624-634.

[14] U. Guldener, S. Heck, T. Fiedler, J. Beinhauer, J. H. Hegemann, A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. 24 (1996) 2519-2524.

[15] V. Benes, Z. Hostomsky, L. Arnold, V. Paces, M13 and pUC vectors with new unique restriction sites for cloning. Gene 130 (1993) 151-152.

[16] A Stotz, P. Linder, The *ADE2* gene from *Saccharomyces cerevisiae*: sequence and new vectors. Gene 95 (1990) 91-98.

[17] K. J. Hutter, H. E. Eipel, Microbial determinations by flow cytometry. J. Gen. microbial. 113 (1979) 369-375.

[18] I. M. Fingerman, C. –L.Wu, B. D. Wilson, S. D. Briggs, Global loss of Set1-mediated H3 Lys^4 trimethylation is associated with silencing defects in *Saccharomyces cerevisiae*. 280 (2005) 28761-28765.

[19] K. L. Clark, M. Ohtsubo, T. Nishimoto, M. Goebl, G. F. Sprague Jr, The yeast SRM1 protein and human RCC1 protein share analogous functions. Cell Regul. 2 (1991) 781-

792.

[20] T. Matsumoto, D. Beach, Premature initiation of mitosis in yeast lacking RCC1 or an interacting GTPase. Cell 66 (1991) 347-360.

[21] S. Sazer, P. Nurce, A fission yeast RCC1-related protein is required for the mitosis to interphase transition. EMBO J. 13 (1994) 606-615.

[22] J. C. Mallory, T. D. Petes, Protein kinase activity of Tel1p and Mec1p, two Saccharomyces cerevisiae proteins related to the human ATM protein kinase. Proc. Natl. Acad. Sci. USA 91 (2000) 3730-3734.

[23] D. D'Amours, S. P. Jackson, The Mre11 complex: at the crossroads of DNA repair and checkpoint signaling. Nature Rev. Mol. Cell 5 (2002) 317-327.

[24] D. M. Morrow, D. A. Tagle, T. Shiloh, F. S. Collins, P. Hieter, TEL1, an S. cerevisiae homolog of the human gene mutated in Ataxia Telangiectasia, is functionally related to the yeast checkpoint gene MEC1. Cell 82 (1995) 831-840.

[25] T. Usui, H. Ogawa, J. H. J. Petrini, A DNA damage response pathway controlled by Tel1 and the Mre11 complex. Mol. Cell 7 (2001)1255-1266.

[26] M. F. Schwartz, J. K. Duong, Z. Sun, J. S. Morrow, D. Pradhan, D. F. Stern, Rad9 Phosphorylation Sites Couple Rad53 to the *Saccharomyces cerevisiae* DNA Damage Checkpoint. Mol. Cell 9 (2002) 1055-1065.

[27] A. Kusano, T. Yoshioka, H. Nishijima, H. Nishitani, T. Nishimoto, *S. pombe* RanGAP homolog, SpRna1, is required for centromeric silencing and chromosome segregation. Mol. Biol. Cell 15 (2004) 4960-4970.

[28] K. Shirahige, Y. Hori, K. Shiraishi, M. Yamashita, K. Takahashi, C. Obuse, T. Tsurimoto, H. Yoshikawa, Regulation of DNA-replication origins during cell-cycle progression. Nature 395 (1998) 618-621.

[29] R. Kato, H. Ogawa, An essential gene, *ESR1*, is required for mitotic cell growth, DNA repair and meiotic recombination in *Saccharomyces cerevisiae*. Nucleic Acids Res. 22 (1994) 3104-3112.

Table 1 Yeast strains and Plasmid

Strain	Genotype (Background: *len2-3,112 his3 ura3 trp1 ade2 can1-100*)	Reference & source
N43-6C-GSP1	*MAT***a** *gsp1::HIS3::GSP1::LEU2*	13
N43-6C-gsp1-479	*MAT***a** *gsp1::HIS3::gsp1-479::LEU2*	13
N43-6C-gsp1-1757	*MAT***a** *gsp1::HIS3::gsp1-1757::LEU2*	13
N635-8B	*MAT* *gsp1::HIS3::GSP1::LEU2 mec1::TRP1*	This study
N655-17B	*MAT***a** *gsp1::HIS3::gsp1-16::LEU2 mec1::TRP1*	This study
N656-6A	*MAT***a** *gsp1::HIS3::gsp1-322::LEU2 mec1::TRP1*	This study
N627-16B	*MAT***a** *gsp1::HIS3::gsp1-479::LEU2 mec1::TRP1*	This study
N628-11A	*MAT* *gsp1::HIS3::gsp1-1268::LEU2 mec1::TRP1*	This study
N652-14A	*MAT***a** *gsp1::HIS3::gsp1-1547::LEU2 mec1::TRP1*	This study
N653-1B	*MAT* *gsp1::HIS3::gsp1-1757::LEU2 mec1::TRP1*	This study
YEF578a	*MAT***a** *mec1::TRP1*	Gift from Shirahige
N275-2B	*MAT* *mec1::TRP1*	This study
N741-6B	*MAT* *gsp1::HIS3::gsp1-479::LEU2 mec1::TRP1 RAD53-myc::kanMX*	This study
N740-15B	*MAT***a** *gsp1::HIS3::gsp1-1757::LEU2 mec1::TRP1 RAD53-myc::kanMX*	This study
N739-2D	*MAT* *mec1::TRP1 RAD53-myc::kanMX*	This study
N741-7B	*MAT* *RAD53-myc::kanMX*	This study
TH53	*MAT***a** *RAD53-myc::kanMX*	Gift from Hishida
N266-8D	*MAT***a** *rad50::ADE2*	This study
N667-1B	*MAT* *gsp1::HIS3::gsp1-479::LEU2 mec1::TRP1 rad50::ADE2*	This study
N669-4A	*MAT* *gsp1::HIS3::gsp1-1757::LEU2 mec1::TRP1 rad50::ADE2*	This study
N407-11C	*MAT***a** *tel1::kanMX*	This study
N668-7B	*MAT* *gsp1::HIS3::gsp1-479::LEU2 mec1::TRP1 tel1::kanMX*	This study
N684-8B	*MAT* *gsp1::HIS3::gsp1-1757::LEU2 mec1::TRP1 tel1::kanMX*	This study
YHY301A	*MAT***a** *rad53-1::URA3*	28
N677-5B	*MAT* *gsp1::HIS3::gsp1-479::LEU2 rad53-1::URA3*	This study
N526-1D	*MAT* *gsp1::HIS3::gsp1-1757::LEU2 rad53-1::URA3*	This study
N536-2A	*MAT* *rad9::hisG-URA3-hisG*	This study
N686-4A	*MAT***a** *gsp1::HIS3::gsp1-479::LEU2 rad9::hisG-URA3-hisG*	This study
N536-2B	*MAT* *gsp1::HIS3::gsp1-1757::LEU2 rad9::hisG-URA3-hisG*	This study
N616-1C	*MAT***a** *gsp1::HIS3::gsp1-1757::LEU2 rad9::hisG-URA3-hisG mec1:: TRP1*	This study
N569-7B	*MAT* *gsp1::HIS3::gsp1-1757::LEU2 rad9::hisG-URA3-hisG tel1::kanMX*	This study

Plasmid	Genotype Reference & source	
pRK900	*MEC1*, *URA3*, *CEN4, ARS1*	29
prad50A	*rad50::ADE2* on pUC29*	This study

*: pUC29 was described by Benes *et al*. 1993 [15].

Figure legends

Fig. 1. *gsp1* suppressed HU sensitivity in *mec1* mutant. (A) Allele specific suppression by *gsp1* to HU sensitivity in *mec1* cells. The indicated *mec1* cells, *mec1 GSP1* [N635-8B], *mec1 gsp1-479* [N627-16B], *mec1 gsp1-1268* [N628-11A], *mec1 gsp1-1757* [N653-1B], *mec1 gsp1-16* [N655-17B], *mec1 gsp1-322* [N656-6A] and *mec1 gsp1-1547* [N652-14A], were spotted on synthetic medium without (no drug) or 50 mM HU and then incubated at 26°C for 3 days. [ ] indicates the name of the strain used. (B) Cell-cycle analysis of wild-type and *mec1* cells. Cells were cultured in synthetic medium at 26ºC until they reached an OD660 nm= 0.1 and they were then incubated in the presence of 100 mM HU. At the indicated times, samples were removed and processed for FACS analysis.

Fig. 2. *gsp1* restored the *mec1* cells' sensitivity to DNA damage. The indicated cells were plated onto YPDA medium after appropriate dilution. One plate was not irradiated as a control. The plates, irradiated with UV or inoculated with yeasts treated with MMS, were incubated in a dark book at 27°C for 3 days. The percentage of the survived colonies obtained at various UV doses or times after MMS treatment is shown. Closed triangles: *mec1* [N275-2B]. Open triangles: *GSP1* [N43-6C-GSP1]. Closed squares: *gsp1-479*[N43-6C-gsp1-479]. Open squares: *mec1 gsp1-1268* [N627-16B]. Closed circles: *gsp1-1757* [N43-6C-gsp1-1757]. Open circles: *mec1 gsp1-1757* [N653-1B].

Fig. 3. Phosphorylation of Rad53-myc protein in wild-type and *mec1* cells. The indicated cells, which had *RAD53-myc* fusion gene in their genome genetically derived from strain TH53, were cultivated at 26°C. During exponential growth, i.e., OD_{660nm}= 1.0, half of each yeast culture was treated with 60 J/m^2 UV, and the total cellular extracts were prepared as described

in Materials and Methods. The cellular extracts were separated by SDS-PAGE and subjected to immunoblotting analysis with anti-myc antibody.

Fig. 4. The pathway from Tel1 to Rad53 functioned in the mec1 gsp1 cell. (A) The *TEL1* Gene was required for the suppression of the *mec1* phenotype by *gsp1*. Cells harboring the plasmid pRK900, containing the *MEC1* and *URA3* genes, were spotted on synthetic medium with or without (no drug) 1.0 mg/ml 5FOA, and then incubated at 26°C for 3 days as described in Materials and Methods. The *mec1 gsp1-1757* and *tel1 mec1 gsp1-1757* cells, which survived on the 5FOA plate, were then cultured and spotted on HU plate to examine the *TEL1*-dependency of the HU sensitivity of the *mec1 gsp1-1757* cells. The strains were: *mec1* [N275-2B], *rad50* [N266-8D], *tel1* [N407-11C], *mec1 gsp1-479* [N627-16B], *rad50 mec1 gsp1-479* [N667-1B], *tel1 mec1 gsp1-479* [N668-7B], *mec1 gsp1-1757* [N653-1B], *rad50 mec1 gsp1-1757* [N669-4A], and *tel1 mec1 gsp1-1757* [N684-8B]. [ ] indicates the name of the strain used. (B) The *gsp1* mutations could suppress the HU sensitivity in the *rad9* cells, but not in the *rad53* cells. The indicated cells, *GSP1* [N43-6C-GSP1], *rad53 gsp1-479* [N677-5B], *rad53 gsp1-1757* [N526-1D], *rad53* [YHY301A] (upper panel), *rad9* [N536-2A], *rad9 gsp1-479* [N686-4A], *rad9 gsp1-1757* [N536-2B], *rad9 gsp1-1757 tel1* [N569-7B], and *rad9 gsp1-1757 mec1* [N616-1C] (lower panel), were spotted onto synthetic medium with or without (no drug) 50 mM HU, and then incubated at 26°C for 3 days. [ ] indicates the name of the strain used.

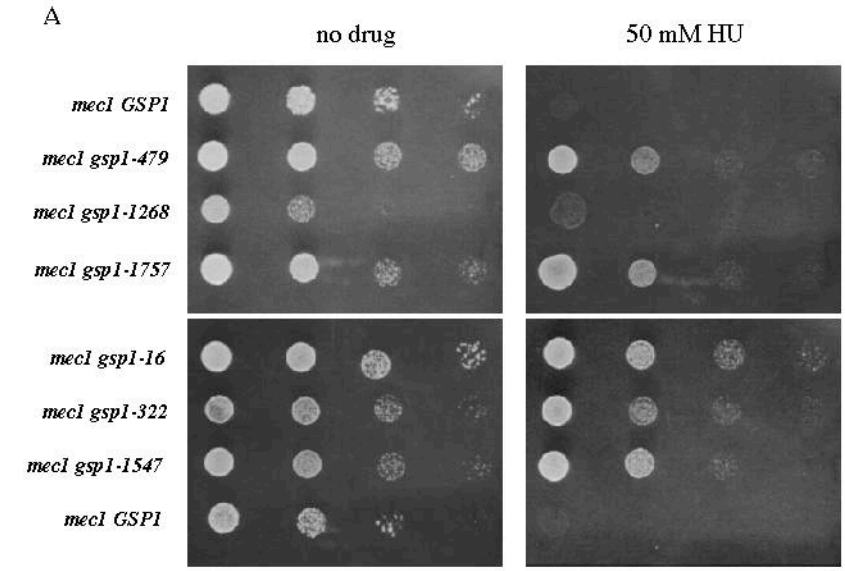
A
no drug
50 mM HU
mec1 GSP1
mec1 gsp1-479
mec1 gsp1-1268
mec1 gsp1-1757
mec1 gsp1-16
mec1 gsp1-322
mec1 gsp1-1547
mec1 GSP1

Fig. 1A

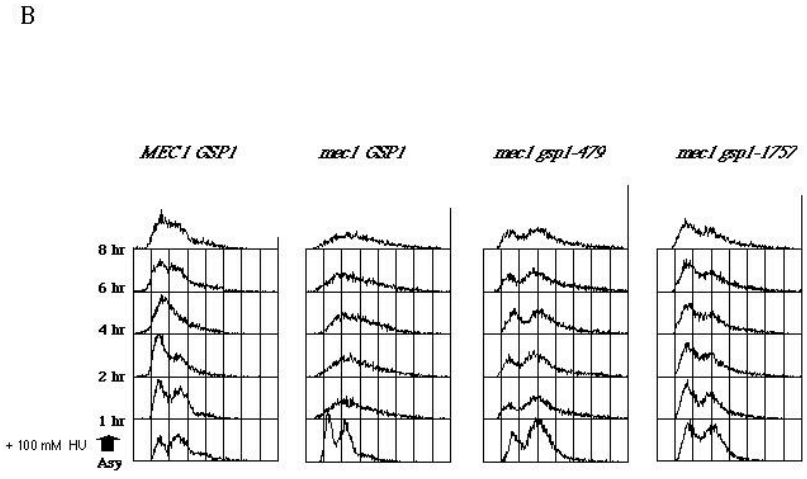
B
MEC1 GSP1
mec1 GSP1
mec1 gsp1-479
mec1 gsp1-1757
8 hr
6 hr
4 hr
2 hr
1 hr
+ 100 mM HU
Asy

Fig. 1B

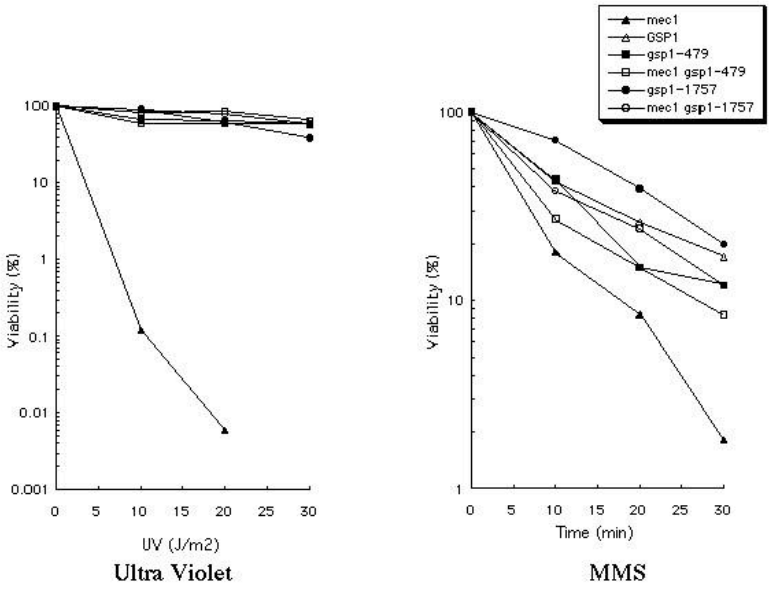
mec1
GSP1
gsp1-479
mec1 gsp1-479
gsp1-1757
mec1 gsp1-1757
Viability (%)
100
10
1
0.1
0.01
0.001
0
5
10
15
20
25
30
UV (J/m2)
Ultra Violet
Viability (%)
100
10
1
0
5
10
15
20
25
30
Time (min)
MMS

Fig. 2

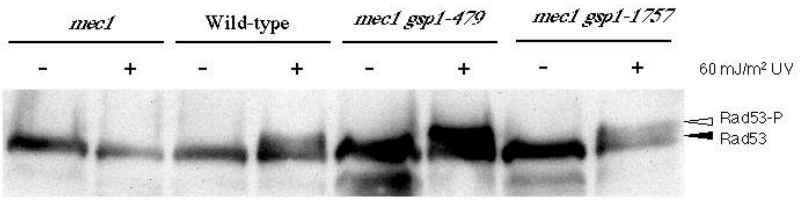
mec1
Wild-type
mec1 gsp1-479
mec1 gsp1-1757
- + - + - + - +
60 mJ/m² UV
Rad53-P
Rad53

Fig. 3

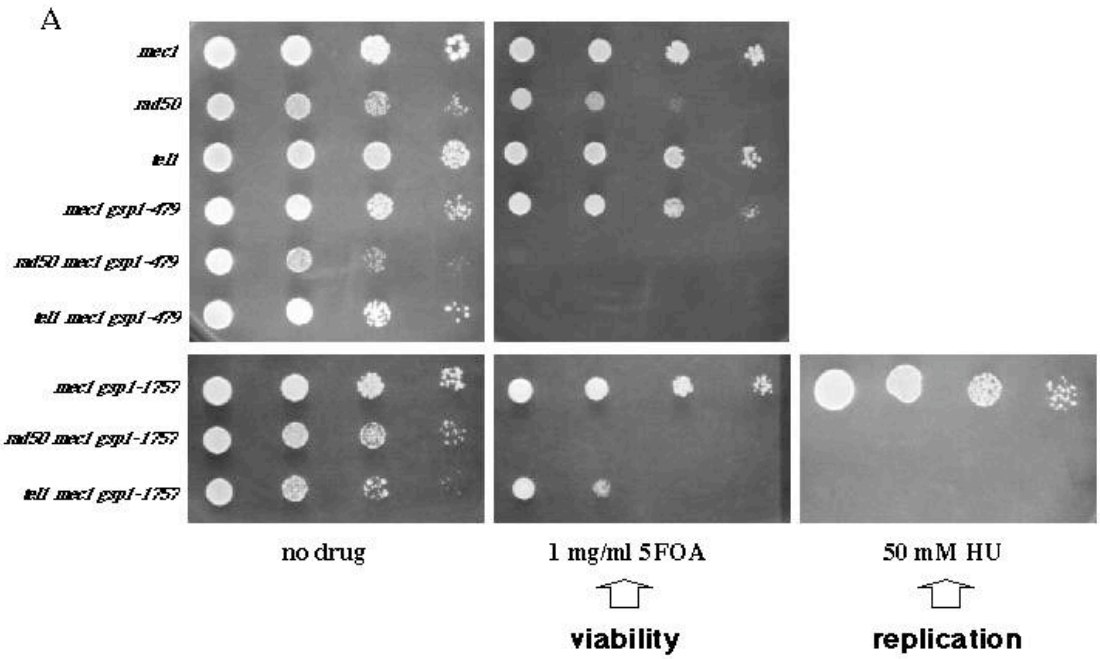
A
mec1
rad50
tel1
mec1 gsp1-479
rad50 mec1 gsp1-479
tel1 mec1 gsp1-479
mec1 gsp1-1757
rad50 mec1 gsp1-1757
tel1 mec1 gsp1-1757
no drug
1 mg/ml 5FOA
50 mM HU
viability
replication

Fig. 4A

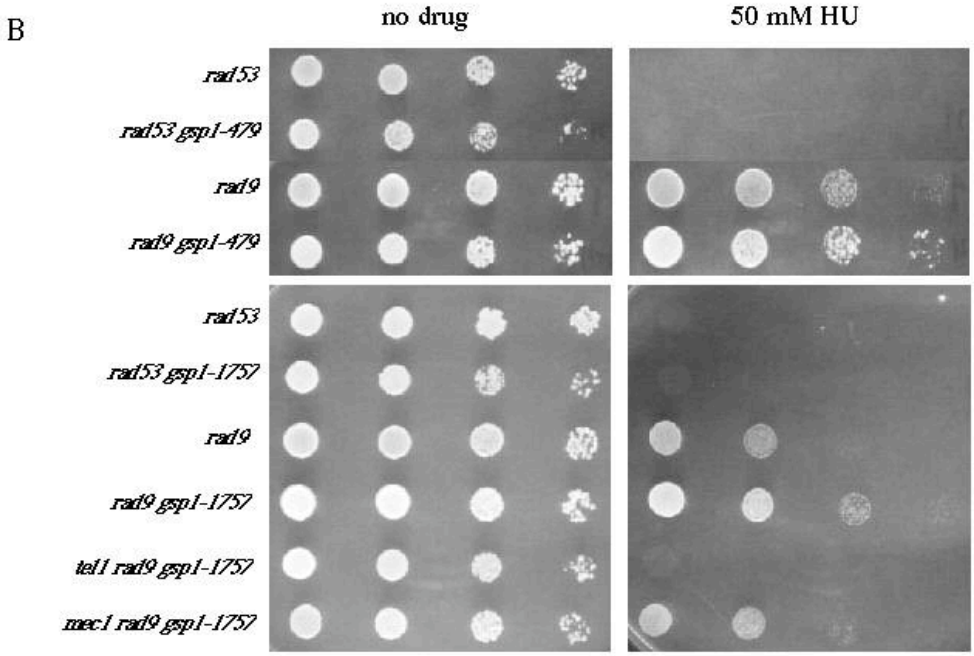
B
no drug
50 mM HU
rad53
rad53 gsp1-479
rad9
rad9 gsp1-479
rad53
rad53 gsp1-1757
rad9
rad9 gsp1-1757
tel1 rad9 gsp1-1757
mec1 rad9 gsp1-1757

Fig. 4B