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Saccharomyces cerevisiae MGS1 is essential in strains deficient in the *RAD6*-dependent DNA damage tolerance pathway

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Saccharomyces cerevisiae **Mgs1 protein, which possesses DNA-dependent ATPase and single strand DNA annealing activities, plays a role in maintaining genomic stability. We found that *mgs1* is synthetic lethal with *rad6* and exhibits a synergistic growth defect with *rad18* and *rad5*, which are members of the *RAD6* epistasis group important for tolerance of DNA damage during DNA replication. The *mgs1* mutant is not sensitive to DNA-damaging agents, but the *mgs1 rad5* double mutant has increased sensitivity to hydroxyurea and a greatly increased spontaneous mutation rate. Growth defects of *mgs1 rad18* double mutants are suppressed by a mutation in *SRS2*, encoding a DNA helicase, or by overexpression of Rad52. Moreover, *mgs1* mutation suppresses the temperature sensitivity of mutants in *POL3*, encoding DNA polymerase δ. *mgs1* also suppresses the growth defect of a *pol3* mutant caused by expression of *Escherichia coli* RuvC, a bacterial Holliday junction resolvase. These findings suggest that Mgs1 is essential for preventing genome instability caused by replication fork arrest in cells deficient in the *RAD6* pathway and may modulate replication fork movement catalyzed by yeast polymerase δ.**

Keywords: DNA replication/homologous recombination/*MGS1*/*RAD6* epistasis group

Introduction

DNA-damaging agents cause DNA lesions that can block the progression of DNA polymerases (Friedberg *et al*., 1995). However, the DNA replication fork can also be hindered during normal vegetative growth in the absence of exogenous DNA-damaging agents. For example, the fork may encounter topological stress, tightly bound protein complexes or aberrant DNA structures. These obstacles can block fork progression leading to gaps or double strand breaks, which can be lethal lesions. Cells have developed mechanisms to enable them to survive such potentially lethal situations. Because these fork block sites do not contain damaged DNA on template DNA, it is likely that the stalled replication complexes are released and normal replication restored by an error-free mechanism. In contrast, translesion polymerases act at forks stalled due to DNA lesions and perform error-prone and error-free DNA repair (Woodgate, 1999; Kunz *et al*., 2000). Recently, there has been increasing evidence that the homologous recombination pathway plays an important role in the error-free process. DNA gaps and double strand breaks caused by replication fork arrest are excellent substrates for homologous recombination (Haber, 1999; Cox *et al*., 2000; Kowalczykowski, 2000; Kuzminov, 2001). Thus, DNA replication, repair and recombination systems are coordinated to prevent genome instability caused by DNA replication arrest.

In *Saccharomyces cerevisiae*, Rad6 and Rad18 play central roles in the post-replication repair (PRR) pathway which supports both error-free and error-prone DNA repair (Prakash *et al*., 1993; Friedberg *et al*., 1995; Xiao *et al*., 2000). Rad6 is a ubiquitin-conjugating enzyme (UBC) that forms a stable complex with Rad18, an ATPase that binds single-stranded DNA (Bailly *et al*., 1994, 1997). The *RAD6* subpathway is the error-prone branch of PRR and it involves translesion polymerases that bypass replication-blocking lesions in a process known as translesion DNA synthesis (Liefshitz *et al*., 1998; Xiao *et al*., 2000). In contrast, Rad5 participates in an error-free subpathway of PRR (McDonald *et al*., 1997). Rad5 is a DNA-dependent ATPase that has homology to the SNF2/SWI2 family of helicases (Johnson *et al*., 1994; Pazin and Kadonaga, 1997); however, no helicase activity has been detected in Rad5 (Johnson *et al*., 1994). Rad5 associates with Rad18 and recruits the Mms2–Ubc13 complex to chromatin in response to DNA damage (Ulrich and Jentsch, 2000). Ubc13 is a UBC that forms a stable complex with a non-canonical UBC variant, Mms2 (Broomfield *et al*., 1998; Brusky *et al*., 2000). The cellular targets of Ubc13–Mms2 and the function of Rad5 remain unknown; however, it is likely that these proteins play an important role in error-free PRR.

The phenotype of *rad6* and *rad18* mutants includes hypersensitivity to radiation, slow growth and a high spontaneous mutation rate (Lawrence and Christensen, 1979; Prakash, 1981). This phenotype is suppressed by mutations in *SRS2* (Aboussekhra *et al*., 1989; Schiestl *et al*., 1990), which encodes a DNA helicase with 3′ to 5′ polarity (Rong and Klein, 1993). Recently, it was shown that *srs2* specifically suppresses mutations in the *RAD5*-dependent error-free branch of the *RAD6* pathway (Ulrich, 2001). Suppression by *srs2* requires homologous recombination, suggesting that Srs2 may channel lesions into the error-free Rad5-dependent PRR subpathway. This may prevent aborted recombination repair at stalled replication forks.

Saccharomyces cerevisiae MGS1 (maintenance of genome stability 1) encodes a protein with centrally located homology to the central region of *Escherichia coli*

RuvB, a Holliday junction branch migration protein, and Rfc, the eukaryotic clamp loader protein (Hishida *et al*., 2001). The homologous region includes the Walker A and B, and sensor I and II motif sequences, which are characteristic of the AAA+ class ATPase family (Hishida *et al*., 2001). The *MGS1* orthologues are highly conserved in prokaryotes and eukaryotes (Barre *et al*., 2001; Hishida *et al*., 2001; Kawabe *et al*., 2001). The *S.cerevisiae* Mgs1 protein possesses DNA-dependent ATPase and DNA annealing activities (Hishida *et al*., 2001). The *mgs1Δ* mutant has an increased rate of homologous recombination, which is elevated further when combined with a mutation in *TOP3*, a type IA topoisomerase (Hishida *et al*., 2001). Recently, it was shown that a mouse homologue of Mgs1, Whip1, interacts physically with the N-terminal portion of mouse Werner's syndrome protein (mWrn) and co-localizes with mWrn in the nucleus (Kawabe *et al*., 2001). *WRN* encodes a member of RecQ helicase family, and mutants in *WRN* are tightly associated with the human hereditary disease Werner's syndrome (Yu *et al*., 1996; Gray *et al*., 1997). Werner's syndrome cells have an increased rate of sister chromatid exchange, slow replication and increased sensitivity to 4-nitroquinoline oxide and camptothecin (Shen and Loeb, 2000). These results suggest that Wrn protein is involved in maintaining genome stability during replication. Based on these results, we proposed that Mgs1 may play a role in maintaining proper DNA topology, which is required for genome stability during replication (Hishida *et al*., 2001).

To understand further the biological role of Mgs1, genetic interactions between *MGS1* and *S.cerevisiae* DNA repair genes were characterized by screening for synthetic lethality in the presence of *mgs1Δ*. This screen identified *rad18*. The synthetic lethality of the *mgs1 rad18* double mutant is suppressed by activation of homologous recombination. In addition, an *mgs1* mutation partially suppresses the growth defect in a *pol3* strain. These results suggest that Mgs1 interacts with the DNA replication machinery and plays a role in preventing genomic instability caused by replication fork arrest. This mechanism may provide an alternative pathway that functions when PRR and homologous recombination are impaired.

Results

Isolation of mutants that require Mgs1 for growth

To isolate mutants that require Mgs1 for growth, 120 000 colonies were screened using a red/white colony-sectoring assay. One mutant was isolated and it was called synthetic growth defect with *mgs1Δ* (*sdm1*). Tetrad analysis revealed that the *mgs1Δ sdm1* double mutant is unable to grow at 37°C, and forms very small colonies after 5 days at 30°C on rich agar plates (Figure 1A). The *sdm1* single mutant is highly sensitive to hydroxyurea (HU) and methylmethane sulfonate (MMS), and extremely sensitive to UV light (Figure 1B). Two plasmids were isolated from a yeast genomic library that complement the MMS sensitivity of *sdm1*. These two plasmids also complement the HU and UV sensitivities of the *sdm1* mutant and the growth defect of the *mgs1Δ sdm1* double mutant (data not shown). Both complementing plasmids include a yeast genomic sequence corresponding to the open reading frame (ORF) of the *RAD18* gene (Figure 1C). To determine whether the *sdm1* mutation is allelic to *RAD18*, the *RAD18* region in the *sdm1* mutant was sequenced. A single G to A transition mutation was found at the second base of the 378th codon of *RAD18* (TGG→TAG), substituting an amber nonsense codon for tryptophan (Figure 1D). Therefore, we named the *sdm1* mutant allele *rad18-378*. *mgs1Δ* was combined with a *rad18* null allele, and heterozygous diploid cells were subjected to tetrad analysis. The *mgs1Δ rad18Δ* double mutant did not grow at 37°C and formed extremely small colonies at 30°C (data not shown).

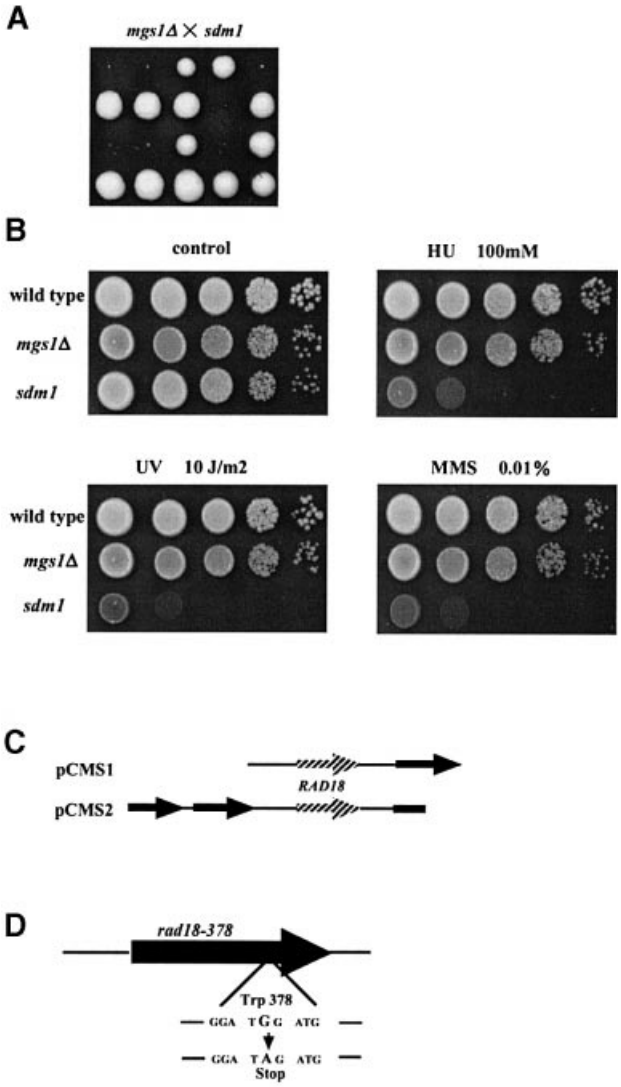

Fig. 1. Identification of the *sdm1* mutation. (**A**) Growth defect of the *mgs1Δ sdm1* double mutant. Tetrad analysis of a cross between *mgs1Δ* and *sdm1*. All tiny colonies were *mgs1Δ sdm1* double mutant. *mgs1Δ* and *sdm1* mutations were confirmed by Leu+ prototropy and UV-sensitive phenotype, respectively. (**B**) Phenotypes of the *sdm1* mutant with respect to growth and sensitivity to MMS, HU and UV. Wild-type, *mgs1Δ* and *sdm1* cells were serially diluted and spotted onto YPAD plates containing 0.01% MMS or 100 mM HU. For UV sensitivity, plates on which cells were spotted were irradiated and incubated at 30°C for 3 days. (**C**) DNA inserts that complement the MMS sensitivity of the *sdm1* mutant. (**D**) *sdm1* mutation and wild-type sequence. Sequence analysis revealed that *sdm1* is an allele of *RAD18*; a nonsense codon substitutes for tryptophan codon 378.

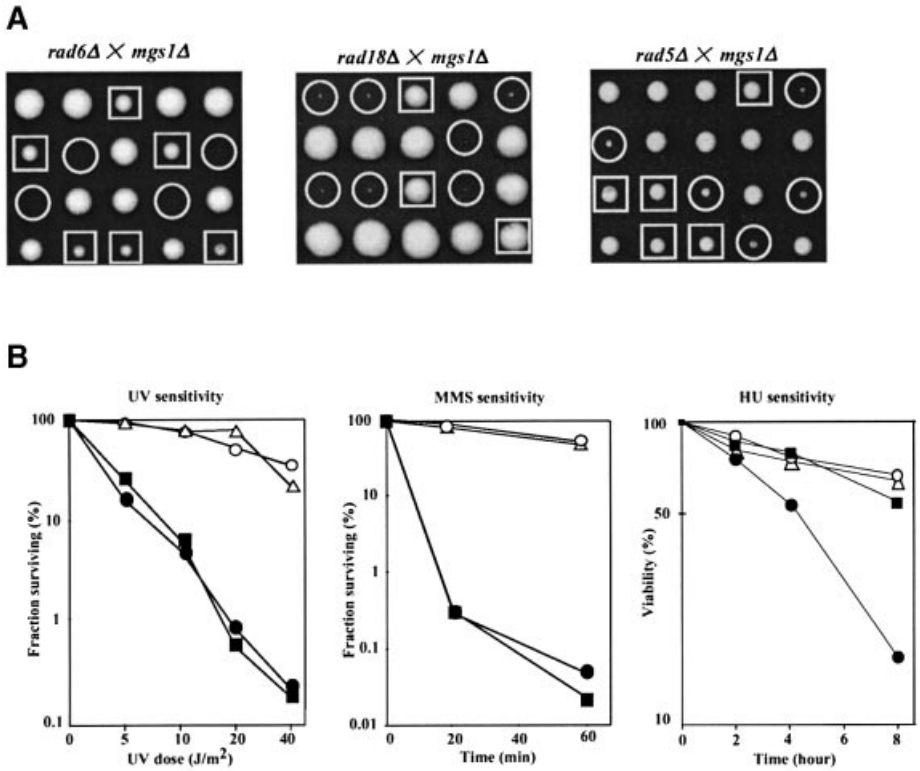

Fig. 2. Synergistic growth defect due to *mgs1Δ* and mutants belonging to the *RAD6* epistasis group. (**A**) Tetrads of diploid cells formed by the crosses indicated were dissected and grown on YPAD at 30°C for 4 or 5 days. *mgs1Δ* was synthetic lethal with *rad6Δ*, and showed a strong synergistic growth defect with *rad18Δ* and *rad5Δ*. Squares indicate the *rad6Δ*, *rad18Δ* and *rad5Δ* mutants. Circles indicate the *rad6Δ mgs1Δ*, *rad18Δ mgs1Δ* and *rad5Δ mgs1Δ* double mutants. (**B**) UV, MMS and HU sensitivity of the *mgs1Δ rad5Δ* cells. UV, MMS and HU sensitivity was determined as described in Materials and methods. Wild type (open circles); *mgs1* (open triangles); *rad5* (filled squares); and *rad5 mgs1* (filled circles).

Relationship between MGS1 and genes in the RAD6 epistasis group

RAD18 is a member of the *RAD6* epistasis group that contributes to DNA damage tolerance. Because *MGS1* interacts with *RAD18*, *mgs1Δ* was also combined with mutants in other genes in the *RAD6* pathway in a background with a wild-type *RAD5* gene (see Materials and methods). (Experiments reported above were carried out in a *rad5*G535R background.) Tetrad analysis was carried out with diploid strains heterozygous for *mgs1Δ* and a deletion of *rad6*, *rad18*, *rad5*, *rad30*, *rev1*, *rev3* or *rev7*, and the double mutants were examined for UV sensitivity and growth rate. The *mgs1Δ* mutation did not affect UV sensitivity of any mutants or the growth of *rad30Δ*, *rev1Δ*, *rev3Δ* or *rev7Δ* mutants (data not shown). However, an *mgs1Δ rad6Δ* double mutant did not grow at 26, 30 or 37°C (Figure 2A). The *mgs1Δ rad18Δ* double mutant was as defective in growth in this strain background as in the *rad5*G535R background (Figure 2A). A *rad5Δ* mutation also caused a synergistic growth defect in *mgs1Δ*, which was less severe than the growth defect in an *mgs1Δ rad18Δ* strain (Figure 2A). The *mgs1Δ rad18Δ* double mutant was very difficult to study, because it grows very poorly and was unstable, spontaneously producing fast growing colonies. Therefore, the phenotype and DNA repair characteristics of *mgs1Δ* mutants were studied in *rad5Δ* cells.

mgs1Δ is no more sensitive than wild-type yeast to UV, MMS or HU; *rad5Δ* is more sensitive to UV and MMS than wild-type yeast, but it is no more sensitive to HU than wild type (Figure 2B). The *rad5Δ mgs1Δ* mutant has a similar level of sensitivity to UV and MMS as *rad5Δ*, but enhanced sensitivity to HU (Figure 2B). These results suggest that *mgs1* mutants require Rad5, Rad6 and Rad18 for normal growth, but not other genes in the *RAD6* epistasis group. The synergistic sensitivity of the *rad5Δ mgs1Δ* double mutant to HU, but not UV or MMS, suggests that Mgs1 plays a role in preventing HU-induced interference with DNA replication in the *rad5* mutant.

Rad5 has been assigned to the error-free subpathway of PRR, suggesting that Mgs1 may play a redundant role in this process. This possibility was tested by measuring spontaneous reversion and recombination frequencies in an *mgs1* mutant background. Intrachromosomal mitotic recombination between heteroallelic DNA sequences was measured using a *lys2BA::URA3::lys2BG* cassette on chromosome II (see Materials and methods). As shown in Table I, the rate of recombination increased slightly in *mgs1Δ* and *rad5Δ* strains. In the *mgs1Δ rad5Δ* double mutant, Lys^+ recombination frequency increased significantly. The majority of Lys^+ recombinants were Lys^+Ura^-, which result from intrachromatid popout or unequal sister chromatid exchange. In addition, the *mgs1Δ rad5Δ* double mutant had a 284- and 644-fold increase in the *ade2-1* and *trp1-1* reversion frequency, respectively (Table II). These results suggest that Mgs1 is functionally redundant with *RAD5*-dependent error-free PRR. Thus, *MGS1* is likely to

Table I. Rate of intrachromosomal recombination

Genotype	Lys$^+$ Ura$^+$ (relative rec.)	Lys$^+$ Ura$^-$ (relative rec.)
MGS1 RAD5	1.6×10^{-5} (1.0)	1.9×10^{-5} (1.0)
mgs1 RAD5	5.3×10^{-5} (3.3)	10.8×10^{-5} (5.7)
MGS1 rad5	2.3×10^{-5} (1.4)	8.6×10^{-5} (4.5)
mgs1 rad5	9.2×10^{-5} (5.7)	50.2×10^{-5} (26.4)

Recombination rates between the direct repeat sequences in *lys2BA::URA3::lys2BG* of the haploid strains were determined. Recombination rates were calculated according to the median method described by Lea and Coulson (1949).

Table II. Spontaneous reversion frequency at *trp1-1* and *ade2-1* alleles

Reversion frequency/10^7 viable cells (fold increase) at 30°C		
	Trp$^+$	Ade$^+$
Wild type	2.0 (×1)	3.6 (×1)
mgs1	8.5 (×4.2)	8.5 (×2.4)
rad5	13.3 (×6.7)	13.3 (×3.7)
mgs1 rad5	1288.2 (×644)	1025.1 (×284)
Reversion frequency/10^7 viable cells (fold increase) at 25°C		
Wild type	1.2 (×1)	1.5 (×1)
mgs1	5.9 (×4.9)	4.3 (×2.8)
pol3-13	6.3 (×5.3)	3.2 (×2.1)
mgs1 pol3-13	43.9 (×36.4)	13.9 (×9.2)

The reversion frequency was determined as described in Materials and methods. The data are the averages of five independent measurements.

play a role in maintaining genomic stability during replication.

Isolation of an mgs1 mutation that confers temperature sensitivity on rad18Δ

An *mgs1* mutant, *mgs1-18*, was isolated that makes *rad18Δ* cells temperature sensitive for growth. In *mgs1-18*, both the 394th lysine codon (AAG) and the 411th glycine codon (GGG) are changed to that for arginine (AGG). Each of the two mutations, K394R and G411R, confer a modest degree of temperature sensitivity for growth on the *rad18Δ* strain (data not shown). As shown in Figure 3A, the *mgs1-18 rad18Δ* double mutant is severely defective in growth at 37°C (Figure 3A). The *mgs1-18 rad18Δ* cells were grown to early logarithmic phase (2–5 × 10^6 cells/ml) in liquid YPAD at 26°C. When the culture was shifted to 37°C, the double mutant ceased to grow after ~5 h (Figure 3B).

Effect of mgs1 on cell cycle progression in a rad18 mutant

Cell cycle progression was analysed in wild-type, *mgs1-18*, *rad18Δ* and *mgs1-18 rad18Δ* cells using fluorescence-activated cell sorting (FACS) analysis to follow cellular DNA content. Asynchronous cultures were grown to early logarithmic phase at 26°C and shifted to 37°C for 6 h. As shown in Figure 4A, the cell cycle of wild-type, *mgs1-18* and *rad18Δ* cells was unaffected by incubation at 37°C and had similar profiles. However, the *mgs1-18 rad18Δ* double mutant accumulated cells with 2C DNA

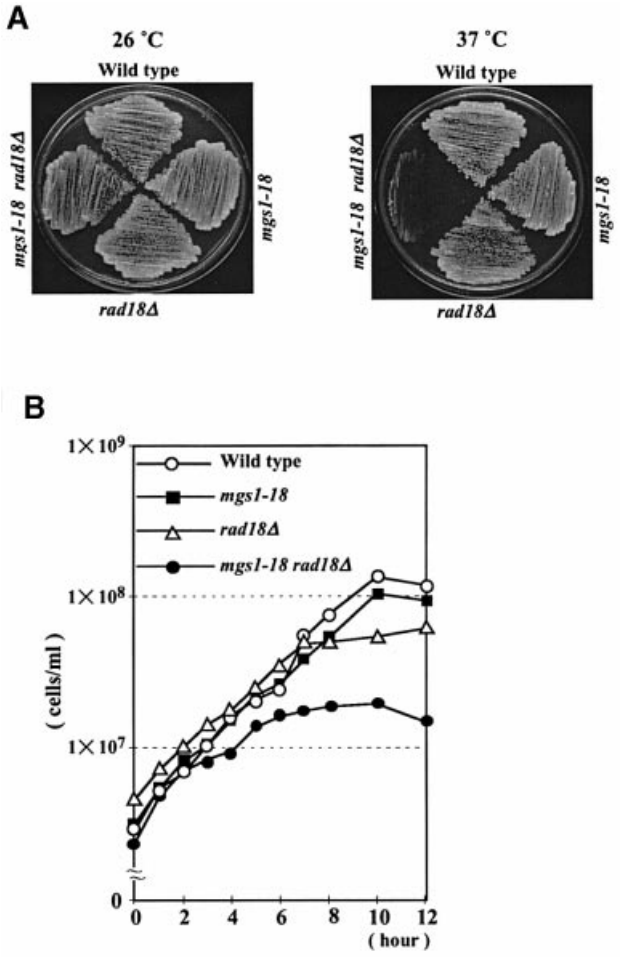

Fig. 3. Temperature sensitivity of *mgs1-18 rad18Δ*. (**A**) Wild-type, *mgs1-18*, *rad18Δ* and *mgs1-18 rad18Δ* cells were streaked onto a YPAD plate and incubated at 30 (left) and 37°C (right) for 3 days. (**B**) Growth of mutant cells after temperature shift to 37°C. Wild-type, *mgs1-18*, *rad18Δ* and *mgs1-18 rad18Δ* cells were grown in liquid YPAD to early logarithmic phase (2–5 × 10^6 cells/ml) at 3°C, and then shifted to 37°C. Wild-type (open diamonds); *mgs1-18* (filled squares); *rad18Δ* (open triangles); and *mgs1-18 rad18Δ* (filled circles).

content. Cells with <1C and >2C DNA content also accumulated. This is probably due to increased cell death, cellular debris or chromosome fragmentation. DNA synthesis was also examined in synchronized cells carrying *mgs1* and/or *rad18* mutations. As shown in Figure 4B, cells were synchronized with α-factor and then released and incubated at 37°C for the indicated times. The majority of wild-type, *mgs1Δ* and *rad18Δ* cells achieved 2C DNA content within 60 min of release from α-factor. In contrast, *mgs1-18 rad18Δ* cells achieved 2C DNA content 100 min after release from α-factor, indicating that *mgs1-18 rad18* is delayed in S-phase progression.

The morphology of the *mgs1-18 rad18Δ* cells was analysed 6 h after the shift to 37°C (Figure 5): ~30% of wild type, *mgs1-18* and *rad18Δ* cells were large budded (dumbbell-shaped), but >70% of *mgs1-18 rad18Δ* cells were large budded (Figure 5A). In addition, >90% of the large-budded *mgs1-18 rad18Δ* cells accumulated nuclei at the bud necks and had a short mitotic spindle; this morphology is characteristic of cells at late S/G_2 (Figure 5B and C).

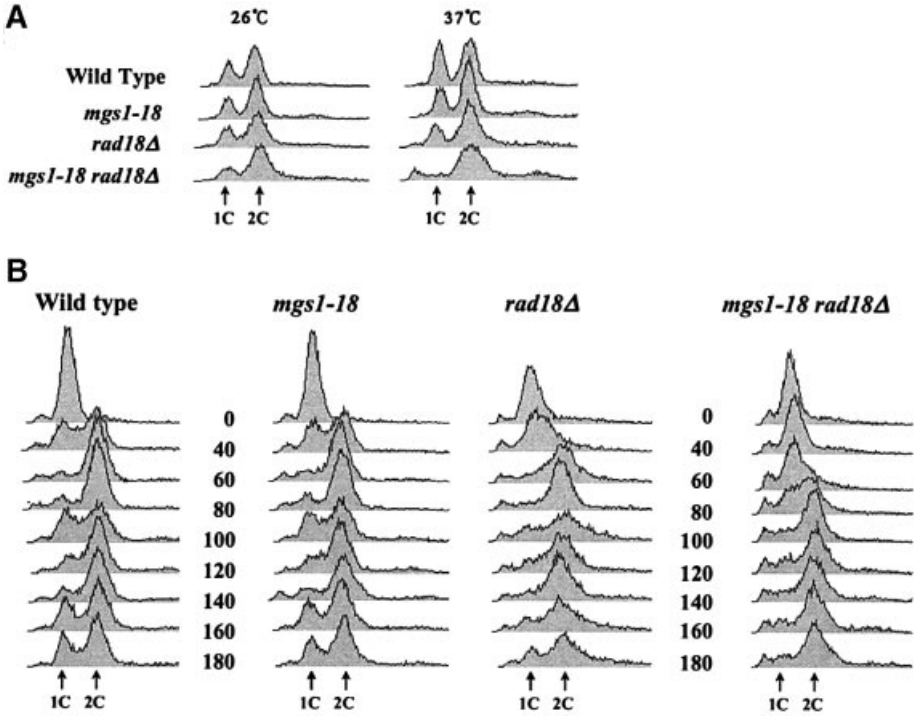

Fig. 4. FACS analysis of *mgs1-18 rad18Δ* cells. (**A**) FACS analysis of the DNA content of asynchronized cells. Cells were incubated to early log phase and then shifted to 37°C for 6 h. (**B**) FACS analysis of the DNA content of synchronized cells. Cells were synchronized at G_1 by treatment with α-factor at 26°C and then released at 37°C. Aliquots were taken at the indicated time points. DNA content was measured by FACS.

Increased homologous recombination suppresses the growth defect of the mgs1 rad18 mutant

Prolonged incubation of *mgs1-18 rad18Δ* cells at the restrictive temperature led to spontaneous accumulation of faster growing cells or colonies. These spontaneous faster growing mutant cells were crossed with the wild-type strain and sporulated. Tetrad analysis revealed that an unlinked mutation was present in these cells that suppresses the UV sensitivity of *rad18Δ* cells. This single mutant displayed moderate UV sensitivity and suppressed the UV sensitivity of both *mgs1-18 rad18Δ* and *rad18Δ* mutants (data not shown). Previous studies showed that *srs2* mutations suppress the UV sensitivity of both *rad6* and *rad18* mutants (Lawrence and Christensen, 1979; Aboussekhra *et al*., 1989; Schiestl *et al*., 1990). Thus, this suppressor mutant was crossed to an *srs2Δ* strain, sporulated and dissected. All 48 spores were moderately sensitive to UV (data not shown), suggesting that this suppressor mutation is tightly linked to *SRS2*. Sequence analysis of the *SRS2* region in the suppressed strain revealed a frameshift at nucleotide 1297 of the *SRS2* ORF, which truncates the Srs2 protein approximately two-thirds of the way from the translation start. *srs2Δ* also suppresses the growth defect of the *mgs1-18 rad18Δ* double mutant at the restrictive temperature (Figure 6A), as well as the poor growth phenotype of the *mgs1Δ rad18Δ* mutant (Figure 6B). The triple mutant grows at the same rate as wild-type yeast.

Genetic interactions were also examined between *mgs1* and other yeast recombination genes including Rad51 and Rad52, which play central roles in homologous recombination. *mgs1-18 rad18Δ srs2Δ* was lethal with *rad52Δ* and *rad51Δ* mutations at 37°C, respectively (data not shown). *mgs1Δ rad18Δ srs2Δ* was also lethal with *rad52Δ* and *rad51Δ* mutations, respectively (data not shown). These results suggest that loss of Srs2 function suppresses the synthetic lethality of the *mgs1 rad18* strain by a mechanism that requires homologous recombination. This idea is consistent with the observation that overexpression of Rad52 suppresses the growth defect of *mgs1-18 rad18Δ* (Figure 6C). In contrast, overexpression of Rad51 does not suppress the lethality of the double mutant at restrictive temperature (Figure 6C). These results suggest that Rad52 is a limiting factor for the growth of the *mgs1 rad18* double mutant. The role of homologous recombination for suppression of the growth defect was examined in an *mgs1-18 rad18Δ rad51Δ* triple mutant. In this strain background, overexpression of Rad52 did not suppress lethality (Figure 6C), suggesting that this suppression is dependent on homologous recombination function. These results clearly indicate that the lethal situation caused by the *mgs1 rad18* double mutation is rescued by activation of homologous recombination function.

mgs1 suppresses the growth defect of DNA polymerase δ mutants

The above studies examine the genetic interactions between *mgs1* and genes in the *RAD6* epistasis group. The results suggest that Mgs1 is involved in normal DNA replication and an error-free PRR pathway. It is therefore possible that Mgs1 interacts with the DNA replication machinery in yeast. This idea was tested by crossing *mgs1Δ* with DNA replication mutants *pol1-17*, *pol2-11* and *pol3-13*. These mutations are in the genes encoding DNA polymerase α, ε and δ, respectively. *mgs1Δ* did not alter the UV or MMS sensitivity or the growth rate of

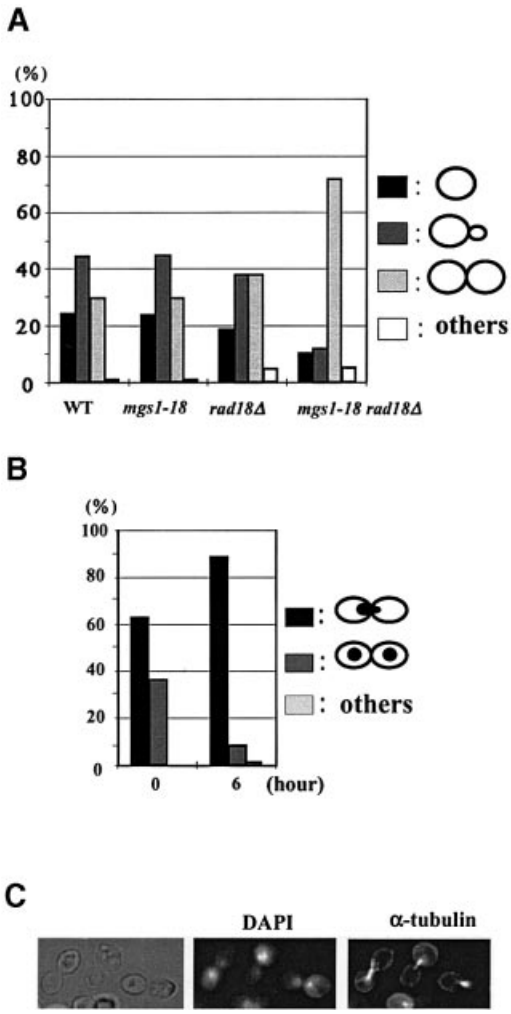

Fig. 5. Morphological analysis of *mgs1-18 rad18Δ* mutants grown at restrictive temperature. Wild-type, *mgs1-18*, *rad18Δ* and *mgs1-18 rad18Δ* cells were grown to early log phase at 26°C, and then grown at 37°C for 6 h. (**A**) Cells were harvested and the shapes were monitored under a microscope. (**B**) *mgs1-18 rad18Δ* double mutant cells (0 and 6 h) were stained with DAPI. At least 200 cells were examined. (**C**) The cells grown at 37°C for 6 h were harvested and fixed in formaldehyde. Nuclear and microtubular structures were visualized with DAPI and anti-tubulin antibodies, respectively.

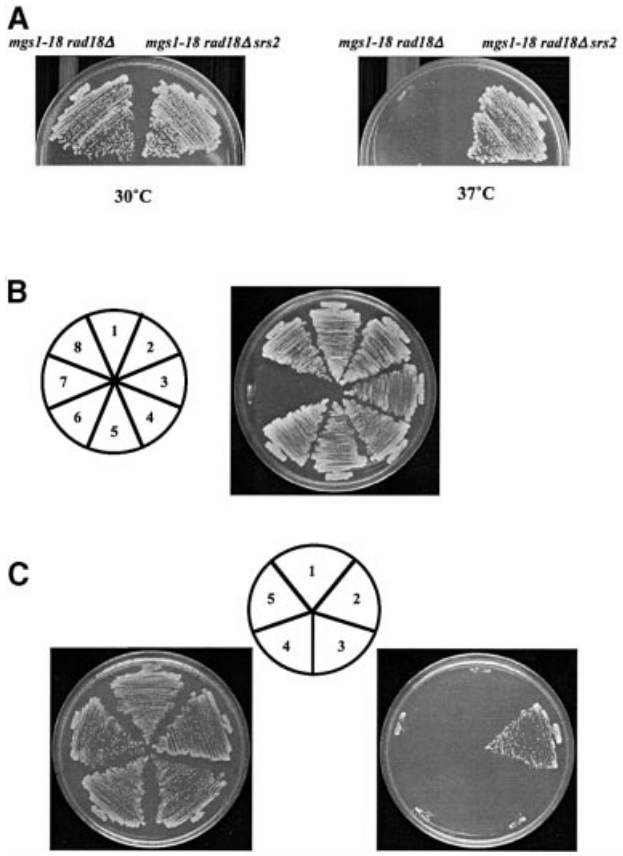

Fig. 6. Suppression of temperature sensitivity of *mgs1-18 rad18*. (**A**) *srs2* mutation suppressed the temperature sensitivity for growth of the *mgs1-18 rad18Δ* mutant. *mgs1-18 rad18Δ srs2* cells were streaked onto YPAD and incubated at 26 (left) or 37°C (right) for 3 days. (**B**) *srs2Δ* mutation suppresses the growth defect of the *mgs1Δ rad18Δ* mutant. Spore colonies resulting from diploid strain TO202 were streaked onto YPAD and incubated at 26°C: (1) wild-type; (2) *mgs1Δ*; (3) *srs2Δ*; (4) *rad18Δ*; (5) *mgs1Δ srs2Δ*; (6) *srs2Δ rad18Δ*; (7) *mgs1Δ rad18Δ*; (8) *mgs1Δ rad18Δ srs2Δ*. (**C**) Increased expression of Rad52p suppresses the growth defect of *mgs1-18 rad18Δ*. *mgs1-18 rad18Δ* cells were transformed with pRS426(1), pSC52 (2) and pSC51 (3). *mgs1-18 rad18Δ rad51Δ* cells were transformed with pRS426 (4) and pSC52 (5). All transformants were streaked onto SC –Ura and incubated at 26 (left) or 37°C (right) for 3 days.

pol2-11 mutant. *mgs1Δ* slightly suppressed the temperature sensitivity of *pol1-17* (data not shown). However, *mgs1Δ* clearly suppressed the growth defect of *pol3-13* at 28 and 30°C (Figure 7A). In addition, overexpression of Mgs1 inhibited the growth of the *pol3-13* mutant (Figure 7B). These results suggest that wild-type Mgs1 interferes with DNA replication in the *pol3-13* mutant. *pol3-13* is a DNA repair-deficient temperature-sensitive allele of *pol3* with a cysteine to serine change in a conserved C-terminal residue (C1074S) (Giot *et al*., 1997). The growth of another *pol3* allele, *cdc2-3*, which is temperature sensitive for growth but not DNA repair deficient, was also partially suppressed by *mgs1Δ* (data not shown). These results suggest that *mgs1* mutation suppresses the DNA synthesis defect in *pol3* mutants. However, *mgs1Δ pol3-13* had a synergistic increase in mutation frequency (Table II), suggesting that Mgs1 prevents genomic instability resulting from a mutation in DNA polymerase δ.

The mgs1 mutation alleviates the growth defect in a DNA polymerase δ mutant expressing E.coli RuvC

Previous studies demonstrated that Holliday junction-like structures form at an increased rate during replication in yeast mutants in DNA polymerase (Zou and Rothstein, 1997). This result implies that mutations in DNA polymerase δ cause the replication fork to stall, leading to the accumulation of Holliday-like structures. This also might indicate that *mgs1* mutation suppresses the formation of Holliday junctions in *pol3* mutants. This idea was tested by expressing *E.coli* Holliday junction-specific endonucleases RuvC and RusA in the *mgs1Δ pol3-13* mutant. To ensure that the bacterial proteins entered the yeast nucleus, an SV40 nuclear localization signal (NLS) was added to the N-terminus of the RuvC and RusA ORFs, respectively. NLS-fused RuvC and RusA complemented the UV sensitivity of an *E.coli ruvC* mutant, indicating that these fusion proteins are functional *in vivo* in *E.coli* (data not shown). NLS-RuvC and NLS-RusA were expressed in

yeast cells under the control of the *GAL1* promoter. Although RusA was a useful tool for detecting the formation of Holliday junctions in fission yeast (Doe *et al*., 2000; Boddy *et al*., 2001), RusA expression severely inhibited cell growth in wild-type budding yeast (data not shown). Overexpression of RuvC had no effect on the growth of wild-type or the *mgs1Δ* strain, and it inhibited the growth of *pol3-13* at the permissive temperature (Figure 7C), consistent with the accumulation of RuvC-cleavable Holliday junction structures in the *pol3* mutant (Zou and Rothstein, 1997). These results suggest that Holliday junctions accumulated at replication forks in a *pol3* mutant are cleaved by RuvC and that this process causes lethal DNA double strand breaks to accumulate. However, as shown in Figure 7C, *mgs1* mutation suppressed this growth inhibition. Thus, a mutation in *MGS1* may prevent the formation of Holliday junctions in a DNA polymerase δ mutant.

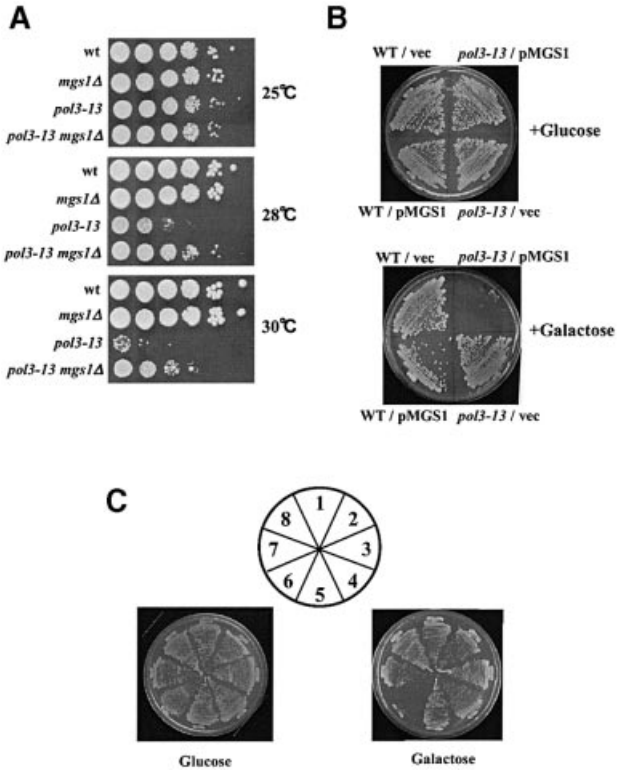

Fig. 7. *Mgs1* interacts genetically with DNA polymerase δ. (**A**) *mgs1* mutation partially suppresses the growth defect caused by *pol3-13* mutation. Wild-type, *mgs1Δ*, *pol3-13* and *mgs1Δ pol3-13* cells were incubated at 25°C. Cells were diluted and spotted onto YPAD plates. The plates were incubated at the indicated temperature for 3 days. (**B**) Overproduction of Mgs1 inhibits the growth of *pol3-13*. Wild-type or *pol3-13* cells harbouring empty vector (vector) or plasmids with the galactose-inducible wild-type *MGS1* gene (pMGS1) were plated onto SC galactose –Leu or SC glucose –Leu plates. Plates were incubated at 25°C for 3 days. (**C**) *mgs1* suppresses the growth inhibition of *pol3-13* cells caused by expression of RuvC. Vector, pSC135; pGA102, NLS-ruvC. (1) Wild-type/vector, (2) wild-type/pGA102, (3) *mgs1Δ*/vector, (4) *mgs1Δ*/pGA102, (5) *pol3-13*/vector, (6) *pol3-13*/pGA102, (7) *mgs1Δ pol3-13*/vector, (8) *mgs1Δ pol3-13*/pGA102.

Discussion

Role of Mgs1 in spontaneously growing cells

This study characterizes genetic interactions between yeast *mgs1* and other yeast DNA repair, recombination and replication genes. In a screen for mutants that are synthetically lethal with *mgs1*, an allele of *rad18*, *rad18-378*, was identified. Additional analysis of double mutants between *mgs1Δ* and genes in the *RAD6* epistasis group showed that *mgs1Δ* causes a synergistic growth defect with *rad5Δ* and synthetic lethality with *rad6Δ* (Figure 2A). Increased homologous recombination promoted by *srs2* mutation or Rad52 overexpression rescues the growth defect in *mgs1-18 rad18Δ* at the restrictive temperature (Figure 6). In addition, *mgs1Δ* partially improves the growth of cells with temperature-sensitive alleles of *POL3* (Figure 7A). Although *mgs1Δ* causes a severe growth defect with *rad5Δ*, the *rad5Δ mgs1Δ* double mutant has a similar level of sensitivity to UV and MMS as the *rad5Δ* single mutant (Figure 2B). However, *rad5Δ mgs1Δ* has increased HU sensitivity and spontaneous mutation and recombination frequencies (Tables I and II). HU does not directly damage DNA, but inhibits DNA synthesis by depleting nucleotide pools, which leads to replication fork arrest. These results suggest that the growth defect of *rad5Δ mgs1Δ* is due to a defect in processing stalled replication forks, but not a defect in DNA repair. These results suggest that Mgs1 plays a pivotal role in preventing genome instability caused by replication fork arrest, which may be an alternative to the error-free subpathway of *RAD6*-dependent PRR.

mgs1-18 rad18Δ cells experience a delay in S-phase progression at the restrictive temperature, and are large budded with undivided nuclei and short spindles (Figures 4 and 5), indicating that these cells arrest at late S/G_2 at the restrictive temperature. Similar characteristics have been observed in leaky DNA synthesis mutants such as *pol1*, *rfc1* and *rfc2* (Lucchini *et al*., 1990, 1988; Zou and Stillman, 1997; Noskov *et al*., 1998). These results indicate that completion of DNA replication is blocked in *mgs1-18 rad18* cells, even though the bulk DNA content is doubled eventually. In addition, *srs2* suppresses the severe growth defect of *mgs1Δ rad18Δ* and *mgs1-18 rad18Δ* (Figure 6A and B). *srs2* also suppresses the slow growth and hypermutation phenotypes of *rad5 mgs1* (data not shown). *srs2* was first identified as a suppressor of the UV sensitivity of *rad6* and *rad18* mutants (Lawrence and Christensen, 1979; Aboussekhra *et al*., 1989). The suppression is specific for the *RAD5*-dependent error-free branch of the *RAD6* pathway (Ulrich, 2001). Therefore, *srs2* suppresses *mgs1-18 rad18* by bypassing *RAD6*-dependent PRR.

Previous studies show that *srs2* suppression of *rad6* and *rad18* depends on the *RAD52* homologous recombination pathway, possibly by channelling aborted repair events into recombinational repair (Schiestl *et al*., 1990). However, the *rad6 srs2 rad52* triple mutant, which is defective in both PRR and homologous recombination, is not lethal, indicating that there is an alternative pathway for reinitiating replication at an arrested replication fork. This study shows that the *mgs1 rad18 srs2* triple mutation, which does not cause a growth defect, is a synthetic lethal with *rad52Δ* and *rad51Δ* mutations. In addition, overexpression of *RAD52* rescues the lethality of *mgs1-18 rad18Δ* mutants by increasing the level of homologous recombination activity (Figure 6C). This result indicates

that the *RAD52* homologous recombination pathway is essential for the growth of the *mgs1 rad18* mutant, possibly by restoring replication from arrested replication forks in a recombination-dependent manner. These results demonstrate that Mgs1 participates in a novel error-free pathway, which may play an essential role in restoring replication from arrested replication forks when *RAD6*-dependent PRR and homologous recombination pathways are impaired.

Functional interaction between Mgs1 and DNA polymerase δ

Previous studies show that *mgs1* increases recombination between repeated sequences and causes a synergistic growth defect in a *top3* (topoisomerase III) strain (Hishida *et al*., 2001). It was also shown previously that overproduction of Mgs1 makes cells sensitive to HU and the topoisomerase I inhibitor camptothecin (Hishida *et al*., 2001). These results suggest that Mgs1 influences DNA topology during replication and plays a role in DNA replication. This study shows that *mgs1Δ* suppresses the temperature sensitivity for growth of mutants of DNA polymerase δ (Figure 7A). *mgs1Δ* also synergistically increases the mutation frequency in a *pol3* mutant (Table II). These results suggest that deficiency of Mgs1 allows replication fork progression in the presence of the mutant DNA polymerase δ at the expense of replication fidelity. Mgs1 may regulate the processivity and fidelity of replication catalysed by polymerase δ. Expression of RuvC specifically inhibits the growth of *pol3-13* at the permissive temperature but does not affect the growth of wild-type and *mgs1Δ* cells (Figure 7C). This is consistent with the suggestion that RuvC-cleavable Holliday junction structures accumulate in a DNA polymerase δ mutant (Zou and Rothstein, 1997). *mgs1Δ* suppresses RuvC-mediated growth inhibition of *pol3-13* mutants (Figure 7C), indicating that RuvC resolvase inhibits the growth of DNA polymerase δ mutants in an Mgs1-dependent manner. Mgs1 deficiency may suppress the accumulation of Holliday junctions, and wild-type Mgs1 may promote formation of these structures in a *pol3* mutant. Mgs1 possesses DNA annealing activity and affects DNA topology (Hishida *et al.*, 2001); thus, it is possible that Mgs1 is required for regression of the replication fork, which facilitates formation of a Holliday junction at a stalled replication fork. However, it has not been proven that the *E.coli* Holliday junction resolvases exclusively target Holliday junction structures in yeast (Doe *et al*., 2000; Boddy *et al*., 2001), and this hypothesis needs rigorous testing.

The *MGS1* orthologues are highly conserved from bacteria to human (Barre *et al*., 2001; Hishida *et al*., 2001; Kawabe *et al*., 2001), suggesting that Mgs1 performs an important biological function. Evidence shown here indicates that the biological function of Mgs1 is related to DNA replication. If that is the case, it can be asked why a deficiency in Mgs1 does not cause lethality or sensitivity to UV, HU and MMS. When DNA replication forks proceed, they encounter endogenous DNA obstacles such as DNA topological stress, tightly bound proteins or specific DNA sequences and structures that inhibit DNA synthesis. Mgs1 may act to avoid fatal replication arrest caused by these obstacles. It is possible that Mgs1 is required to overcome replication blocks involving polymerase δ, which is redundant with the function of genes in the *RAD6* epistasis group (Figure 8). In addition, in the absence of Mgs1 and Rad6, DNA replication can be restored at a blocked replication fork by homologous recombination only when recombination activity is induced or activated (i.e. Rad52 overexpression or *srs2* mutation) (Figure 8). Mgs1 is essential for growth when PRR and homologous recombination pathways are impaired; this suggests that Mgs1 participates in an alternative pathway which restores replication at blocked replication forks involving DNA polymerase δ. The mouse orthologue of *MGS1*, Whip1, physically interacts with Werner syndrome protein (WRN), which belongs to the bacterial RecQ helicase family (Kawabe *et al*., 2001). Human WRN physically interacts with the DNA replication complex and stimulates DNA synthesis by polymerase δ (Lebel *et al*., 1999; Kamath-Loeb *et al*., 2000), implicating the WRN protein in restoring replication fork movement after DNA damage or topological stress. Hence, Mgs1 orthologues and RecQ helicases may act in a coordinated manner to regulate fork progression by DNA polymerase δ and may play a role in restoring replication at stalled forks.

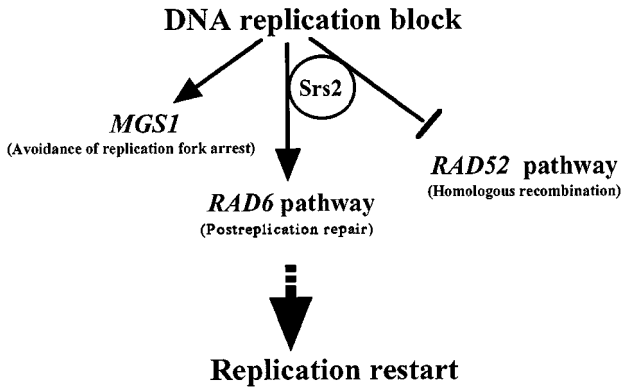

Fig. 8. Proposed role of Mgs1 in resolving stalled replication forks. In *rad6Δ* cells, Mgs1 is essential for restoration of replication fork arrest. In the absence of Mgs1 and Rad6, homologous recombination proteins can relieve the block to replication and allow replication fork progression to resume. Mgs1 might facilitate fork progression catalysed by DNA polymerase δ in a *RAD6* group-independent manner by rescuing the stalled replication forks. The T-bar sign indicates the processes suppressed by functions of the gene products.

In summary, Mgs1 may influence progression of DNA polymerase δ-dependent replication forks and play a role in preventing genomic instability during DNA replication. The present findings provide important clues to understanding the complex interplay between DNA replication, repair and recombination functions, especially at stalled replication forks.

Materials and methods

Strains and plasmids

Yeast strains are listed in Table III. The W303 strain and its derivatives carry a point mutation in the *RAD5* gene, known as the *rad5-535* allele (Fan *et al*., 1996). To avoid the possible effect of the mutation on the phenotypes we intended to study, we used strain W1588, in which the *rad5-G535R* allele of W303 has been replaced by the wild-type *RAD5* gene. The strains derived from W1588, M31 (*rad6*), M35 (*rad18*) and C22 (*rad5*) were kindly provided by R.Woodgate (McDonald *et al*.,

Table III. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Origin
W303-1A	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 rad5-G535R*	Thomas and Rothstein (1989)
W303-1B	*MAT*α *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 rad5-G535R*	Thomas and Rothstein (1989)
TH102-1A	W303-1A, ade3::hisG	laboratory collection
TH102-1B	W303-1B, ade3::hisG	laboratory collection
TH103	TH102-1A, *mgs1::LEU2*	this study
TH104	TH102-1B, *rad18–378*	this study
TH105	TH102-1A, *mgs1::LEU2 rad18-378*	this study
TH106	TH102-1A, *mgs1::LEU2 rad18::TRP1* [pSL1(ARS/CEN *ADE3 URA3 MGS1*)]	this study
TD100	TH103 × TH104	this study
W1588-4A	W303-1A, *RAD5*	McDonald *et al*. (1997)
W1588-4B	W303-1B, *RAD5*	McDonald *et al*. (1997)
M31	W1588-4B, *rad6::LEU2*	McDonald *et al*. (1997)
M35	W1588-4B, *rad18::LEU2*	McDonald *et al*. (1997)
C22	W1588-4B, *rad5::HIS3*	McDonald *et al*. (1997)
TH201	W1588-4A, *mgs1::LEU2*	this study
TH202	W1588-4A, *mgs1::HIS3*	this study
TH203	W1588-4A, *mgs1-18*	this study
TH205	W1588-4A, *rad5::HIS3 mgs1::LEU2*	this study
TH210	W1588-4A, *rad18::TRP1*	this study
TH211	W1588-4A, *rad51::hisG-URA3-hisG*	this study
TH212	W1588-4A, *rad52::TRP1*	this study
TH250	W1588-4A, *lys2BA::URA3lys2BG*	this study
TH251	W1588-4A, *mgs1::LEU2, lys2BA::URA3lys2BG*	this study
TH252	W1588-4B, *rad5::HIS3, lys2BA::URA3lys2BG*	this study
TH253	W1588-4A, *rad5::HIS3 mgs1::LEU2, lys2BA::URA3lys2BG*	this study
TH221	W1588-4A, *mgs1-18 rad18::TRP1*	this study
TH222	W1588-4A, *mgs1-18 rad18::TRP1 rad51::hisG*	this study
TH223	W1588-4A, *mgs1-18 rad18::TRP1 srs2*	this study
TH230	W1588-4A, *mgs1-18 rad18::TRP1 srs2::HIS3*	this study
TH231	W1588-4A, *mgs1-18 rad18::LEU2 srs2::HIS3 rad52::TRP1*	this study
TH232	W1588-4A, *mgs1-18 rad18::TRP1 srs2::HIS3 rad51::hisG-URA3-hisG*	this study
TH250	W1588-4A, *srs2::HIS3*	this study
TH251	W1588-4A, *srs2::HIS3 rad18::TRP1*	this study
TH252	W1588-4A, *mgs1::LEU2 srs2::HIS3*	this study
TH253	W1588-4A, *mgs1::LEU2 srs2::HIS3 rad18::TRP1*	this study
TH260	W1588-4A, *pol3-13.URA3*	this study
TH261	W1588-4A, *mgs1::LEU2 pol3-13.URA3*	this study
TD001	W1588-4A × W1588-4A	this study
TD106	TD001, *MGS1/mgs1::HIS3 RAD6/rad6::LEU2*	this study
TD107	TD001, *MGS1/mgs1::LEU2 RAD5/rad5::HIS3*	this study
TD118	TD001, *MGS1/mgs1::LEU2 RAD18/rad18::TRP1*	this study
TO202	TD001, *MGS1/mgs1::LEU2 RAD18/rad18::TRP1 SRS2/srs2::HIS3*	this study

1997). Strains, *pol1-17* and *pol2-11*, which are W303 derivatives, were gifts from A.Sugino. Strains C23-9B (*cdc2-3*) were kindly provided by L.Prakash. W23 was obtained after five back-crosses between C23-9B and the W1588-4B isogenic strain. All yeast manipulations were as described (Sherman and Hicks, 1986). 5-fluoro-orotic acid (5-FOA) was used to select Ura$^-$ cells as described (Rose and Hieter, 1990).

To construct the *mgs1::HIS3* allele, plasmid pDRH1 was constructed by replacing the *Afl*II–*Xho*I fragment in the *MGS1* coding region with the *HIS3* gene. This plasmid was linearized by digestion with *Fsp*I and transformed into yeast to generate a strain with deletion of nucleotides from +234 to +1665 in the 1761 bp coding region of the *MGS1* gene. The cassette to be used for construction of a *rad18::TRP1* disrupted gene on the chromosome was made as follows. The *TRP1* gene of Ydp-W (Berben *et al*., 1991) was amplified using the primers RAD18F (5′-ATGGACCACCAAATAACCACTGCAAGCGACTTCACGACTACTTCAATACCGGACGGCCAGTGAATTCCCGG) and RAD18R (5′-GCATCAGTTGAATCTTCGTTAGGCAAATTTCTTTCACCAGCAACTCTATCAAGCTAGCTTGGCTGCAGGT) to give a product that contains the *TRP1* gene flanked by the 50 and 51 bp sequences which are identical to the N- or C-terminal parts of the *RAD18* coding region, respectively. Plasmid *srs2Δ::HIS3* was constructed by amplifying the coding region of *SRS2* by PCR using the genomic DNA as template and replacing the *Xba*I fragment of the *SRS2* coding region with a *HIS3* cassette in pUC19. *pol3-13.URA3* was constructed as follows. A 1500 bp PCR fragment containing the C-terminal part of *POL3* with a flanking region was inserted into pRS306 (Sikorski and Hieter, 1989) and Cys1074 was altered to serine with PCR-mediated site-directed mutagenesis to produce pOL302. pOL302 was digested with *Tth*111I and integrated into the genome to produce the *pol3-13* strain. Plasmid containing the *rad51*::*hisG-URA3-hisG* was kindly provided by A.Shinohara (Shinohara *et al*., 1992). Plasmid pSL1 was constructed by inserting the 3.9 kb *Hin*dIII–*Pst*I fragment containing the *MGS1* gene into *Hin*dIII–*Pst*I-digested pDS1 vector (*ADE3 URA3* CEN/ARS). Plasmid pH951 was constructed by inserting the 4.8 kb *Hin*dIII genomic fragment containing the *MGS1* gene into the multicloning site of pUC19 vector. Plasmids pSC52 and pSC51 were constructed by subcloning the *Sal*I fragment containing the *RAD52* gene from Yrp7 (Adzuma *et al*., 1984) and the *Bam*HI fragment containing the *RAD51* gene from YEP-51 (Shinohara *et al*., 1992) into a vector, pRS426 (Stratagene), respectively. To construct the NLS-RuvC and NLS-RusA genes, *ruvC* and *rusA* coding regions amplified by PCR were fused in-frame to their 5′ end with SV40 NLS that have been added to the *GAL4* activation domain sequence. NLS-ruvC and NLS-rusA were inserted into the galactose-inducible vector, p423GAL1 (Mumberg *et al*., 1994), to produce pGA102 or pGA153, respectively. The DNA sequences of the PCR-amplified fragments were confirmed by sequencing the appropriate regions.

Isolation of sdm mutants

Strain TH103-1A (*MAT***a** *mgs1::LEU2 ade2 ade3 leu2 trp1 ura3*) was transformed with the plasmid pSL1 (*MGS1 ADE3 URA3* CEN/ARS). Cells from one of the transformed clones were grown at 30°C in selective

medium to logarithmic phase (1×10^7 cells/ml) and treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (35 μg/ml) at 30°C for 30 min. This treatment reduced the cell viability to ~10%. The cells were washed twice with TE buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA), plated onto YPD plates and incubated for 5 days at 30°C. About 120 000 colonies formed on YPD plates were screened. Non-sectored colonies were patched onto YPD plates, and replica-plated onto 5-FOA plates to confirm the dependence on plasmid pSL1 for viability. Mutants that did not grow or grew poorly on 5-FOA plates were crossed with the wild-type strain (TH102-1B) or *mgs1Δ* strain (TH103-1B) to determine whether the synthetic lethality was caused by a single locus mutation.

Sensitivity to UV light, HU and MMS

A quantitative assay for HU sensitivity was performed at 30°C in YPAD. Overnight cultures were inoculated into fresh YPAD at a 100-fold dilution and cells were allowed to grow at 30°C. HU was added to a final concentration of 100 mM and aliquots were taken at the indicated times. Cells were washed, diluted and plated on YPAD. The cell numbers were counted under a phase contrast microscope. Cell numbers versus colony-forming units of cells were quantified. A quantitative assay for MMS and UV sensitivity was performed as described by Interthal and Heyer (2000). Plates were incubated at 30°C for 3 days in the dark.

Reversion and recombination frequency

For determining reversion frequency of *trp1-1* and *ade2-1* alleles, strains were streaked out on YPAD plates and grown at 30 or 25°C as indicated. Five colonies for each strain were suspended in YPAD medium, grown for 12 h and plated at the appropriate dilution to determine the total cell number on YPAD plates, and the number of Trp^+ or Ade^+ revertants on synthetic complete plates plus 2% glucose (SC glucose) minus Trp, or SC glucose minus Ade plates, respectively. Plates were incubated in the dark for 5 days (–Trp plates) or 7 days (–Ade plates). Trp+ or Ade+ revertants per 10^7 survivors were calculated. Recombination rates were monitored in haploid strains containing *lys2BA::URA3::lys2BG* alleles. To construct *lys2BA* and *lys2BG* alleles, the *LYS2* coding sequence was digested with either *Bam*HI or *Bgl*II, blunt-ended and ligated. For determining recombination rates during mitosis, strains were streaked out on YPAD plates and grown at 30°C. Five colonies for each strain were scraped, suspended in YPAD medium, grown for 12 h and plated at appropriate dilutions to determine the total cell numbers on YPAD and the numbers of recombinants on SC glucose –Lys or SC glucose –Lys–Ura plates.

Isolation of mgs1 mutants that are temperature sensitive for growth with the rad18Δ mutation

Mutagenesis of the *MGS1* gene was performed by PCR as described previously (Muhlrad *et al*., 1992; Umezu *et al*., 1998). PCR primers were designed to amplify the 2.4 kb region of pH951. The primers (1) 5′-GAACGGTTCTCATCAGAATTCAATATTGCG and (2) 5′-GCGTAT-CTATGCTCCTCAAGCCTGGCTCA are complementary to the sequence located 400 bp upstream from the initiation codon and 300 bp downstream from the termination codon of the *MGS1* gene, respectively. Primer (1) was designed to introduce an *Eco*RI site. A *Bam*HI site is located 260 bp downstream from the termination codon of the *MGS1* ORF. The PCR product and vector pRS413 (Stratagene) were digested with *Eco*RI and *Bam*HI. They were mixed in a 1:1 molar ratio and co-transformed into TH106 at 26°C. Transformants were selected on SC plates lacking histidine and then replica-plated onto 5-FOA plates to eliminate pSL1 using the plasmid shuffle technique (Boeke *et al*., 1987). To screen *mgs1* mutants that were temperature sensitive for growth, colonies formed on a 5-FOA plate were patched onto a YPAD plate and incubated at 37°C. The *mgs1* mutants that could not grow at 37°C in the *rad18Δ* mutant were selected. The 2.4 kb *Eco*RI–*Bam*HI fragment containing the *mgs1* mutant allele (*mgs1-18*) obtained by PCR mutagenesis was cloned into a yeast integration vector pRS306 to produce pMGS18. pMGS18 was linearized by digestion with *Mun*I and integrated into the yeast genome. Strains that have left the *mgs1-18* allele behind were selected using 5-FOA medium and temperature sensitivity phenotype.

Flow cytometry

To prepare asynchronized cells for analysis by flow cytometry, wild-type, *mgs1-18*, *rad18Δ* and *mgs1-18 rad18Δ* cells were grown in liquid YPAD to early logarithmic phase ($2–5 \times 10^6$ cells/ml) at 26°C, and shifted to a non-permissive temperature, 37°C, for 6 h. Samples were fixed in 70% ethanol at 4°C. The fixed cells, washed and resuspended in 50 mM sodium citrate pH 7.5, were treated with RNase A (0.25 mg/ml) at 50°C for 1 h, and then with proteinase K (1 mg/ml) at 50°C for 1 h. Cells were stained with propidium iodide (16 μg/ml), and incubated for 1 h at 4°C. The DNA content of cells was analysed with a Becton-Dickinson FACScan flow cytometer. To prepare synchronized cells, cells were grown in liquid YPAD to early logarithmic phase ($2–5 \times 10^6$ cells/ml) at 26°C and treated with α-factor (10 μg/ml) for 2 h. After confirming that cells were arrested at G_1 phase under a microscope, cells were washed to remove α-factor and grown in YPAD at 37°C. The aliquots were taken at the indicated time points and processed for flow cytometry.

Morphological analysis

Wild-type, *mgs1-18*, *rad18Δ* and *mgs1-18 rad18Δ* cells were grown in liquid YPAD to early logarithmic phase ($2–5 \times 10^6$ cells/ml) at 26°C, and then grown at 37°C for 6 h. Cells were harvested and stained with 4′,6-diamino-2-phenylindole (DAPI). Cell morphology (unbudded cell, small-budded cell or large-budded cell) was monitored under a microscope. DAPI and immunofluorescence staining were performed as previously described (Sherman and Hicks, 1986; Redding *et al*., 1991).

Other methods

Overproduction of Mgs1 and NLS-RuvC in yeast cells was performed as described previously (Hishida *et al*., 2001).

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