SPANDIDOS PUBLICATIONS

Disruption of protein-protein interaction in the *Mgl-1* oncoprotein

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Abstract. Mammalian homologues of the *Lethal giant larvae* (*Lgl*) tumor suppressor gene have been identified and these homologues can complement the yeast double mutant of *Sop1* and *Sop2*, the yeast homologue of *Lgl*, as reported previously. In the absence of these genes in yeast, cellular viability is affected at restrictive temperature and salt environments. Members of this family contain five or more of the WD-40 repeat motifs, which is known to be involved in protein-protein interaction. In order to investigate the biochemical roles for conserved amino acids within the most conserved WD-40 repeat motif amongst these family members, we generated deletion mutants for five conserved amino acids (G450, H451, D453, W459 and D460) in mouse *Lgl-1* (*Mgl-1*), located between 450-460 amino acids. We found that the deletion mutants of *Mgl-1*, ΔG450 and ΔD453, were not capable of complementing yeast mutants of *Sop1* and *Sop2* at restrictive temperature and high salt environments. These results indicate that the WD-40 repeat motif is important for cellular viability by regulating temperature-sensitivity and salt tolerance in yeast.

Introduction

Lethal giant larvae (*Lgl*), one of the tumor suppressor genes, has been identified as the first recessive oncogene in *Drosophila* and its homologues have been identified in various organisms (1-3). Genetic analysis of *Lgl* revealed that homozygous mutations at the *Lgl* locus in *Drosophila* lead to the neoplastic development in the presumptive adult optic centers of the larval brain and the imaginal discs (4). One of features of the *Lgl* and its homologues is the WD-40 repeat motif (5). Recent investigation demonstrated that mammalian *Lgl* proteins contain 4 to 5 putative WD-40 repeat motifs and contribute to apico-basolateral polarity by regulating basolateral exocytosis (6). It is becoming evident that the WD-40 repeat sequence is involved in various cellular functions including protein-protein interaction, cell cycle regulation (7-12), pre-mRNA processing (13,14), adaptor/regulatory modules in signal transduction (15-17), and cytoskeleton assembly (5). The WD-40 repeat has a 44-60 amino acid residue long sequence that typically contains GH dipeptide 11 to 24 residues from its N-terminus and WD dipeptide at its C-terminus (18).

It has been shown that the yeast homologues *Sop1* and *Sop2*, which can be substituted by mammalian homologues *Rgl-1* and *Bgl-1*, are involved in salt tolerance and temperature-sensitivity (2,3). To explore whether the WD-40 repeat motif of *Mgl-1* is required for the salt tolerance and temperature-sensitivity in yeast, we generated deletion mutant forms of *Mgl-1* in the most conserved WD-40 repeat domain and analyzed its structural and functional conservation in the absence of yeast *Lgl* homologues, *Sop1* and *Sop2*.

Materials and methods

Yeast strains, genotypes and culture. Two *Saccharomyces cerevisiae* strains (provided by Lennart Alder, Göteborg University, Göteborg, Sweden), *W303* (MATa *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1 ura3-1*) and *WKL-23* (MATa *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1 ura3-1 Sop1Δ::LEU2 Sop2Δ::HIS3*) were used in this study. These strains were cultured at 30˚C in YEPD rich medium containing 120 μg/ml adenine hemisulfate or synthetic minimal medium (SD) containing glucose, necessary amino acids and nucleotides. *Escherichia coli* DH5α was also used for cloning and amplification of DNA. Bacterial cultures were grown at 37˚C in LB medium containing antibiotics and supercoiled recombinant plasmid DNA was isolated using a Mini-Prep kit (Bioneer, Korea).

Cloning of Mgl-1 cDNA and mutagenesis. The full length of *Mgl-1* cDNA was obtained from RT-PCR of mouse brain Poly (A^+) RNA generated using primers 5'-CCATGG ATCCCATGATGAAGTTTCGGTTCCGG-3' (forward) and 5'-CGCGACGCGTCCCCAGAAAATCCTTCAC-3'

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Table I. Primers used for RT-PCR and the site-directed mutagenesis for *Mgl-1*.

Gene	Primer sequences forward (F) and reverse (R)	Product size (bp)
Sop1	F 5'-CTGGATGCTAATCGGCCTTC-3' R 5' -GGCTAATGGTCTCAGCCAGG-3'	880
Mgl-1	F 5'-GCTCTGCCCATGTTGCCA-3' R 5'-CTCTGTGCCGAGAAGGCC-3'	834
Act1	F 5'-TGTCACCAACTGGGACGATA-3' R 5'-CCAAACCCAAAACAGAAGGA-3'	584
Mgl-1 (ΔG450)	F 5'-CTGCTGCTCACTCATGAGGATGGC-3' R 5' -GCCATCCTCATGAGTGAGCAGCAG-3'	
Mgl-1 (ΔH451)	F 5'-GCTCACTGGCCAGGAGGGCAC-3' R 5'-GTGCCCTCCTGGCGAGTGAGC -3'	
Mgl-1 (ΔD453)	F 5'-CTGGCCATGAGGGCACTGTGCGG-3' R 5'-CCGCACAGTGCCCTCATGGCCAG-3'	
Mgl-1 (ΔW459)	F 5'-GCACTGTGCGGTTCGACGCCTCTGG-3' R 5'-CCAGAGGCGTCCCTGAGCACAGTGC-3'	
Mgl-1 (ΔD460)	F 5'-GTGCGGTTCTGGGCCTCTGGTGTG-3' R 5'-CACACCAGAGGCCCAGAACCGCAC-3'	

(reverse). To constitutively express the *Mgl-1* gene in *S. cerevisiae*, we subcloned the full length *Mgl-1* into a yeast expression vector, pYX212 carrying the *TPI* promoter (R&D Systems, USA). The *Mgl-1* (ΔG450), *Mgl-1* (ΔH451), *Mgl-1* (ΔD453), *Mgl-1* (ΔW459), and *Mgl-1* (ΔD460) constructs were generated with the QuikChange™ Site-Direct Mutagenesis kit (Stratagene, USA) using primers described in Table I. All constructs were confirmed by restriction analysis and sequencing (Applied Biosystems, USA).

Reverse transcription-polymerase chain reaction. Yeast strains grown to mid-exponential phase (OD_{600}=1.0) in YPED medium were settled by centrifugation, washed with ice-cold water and resuspended in TES solution (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM EDTA). Acid phenol was added in resuspended cell pellets and incubated at 65˚C for 30-60 min. The lysates were then centrifuged and the supernatants were added with 3 M sodium acetate, pH 5.3 to precipitate RNA and ice-cold 100% ethanol. The RNA pellets were resuspended in DEPC-H_2O and incubated with DNase at 37˚C for 15 min. Purified RNAs were immediately converted to cDNA. First-strand cDNA was synthesized from total RNA using SuperScript™ II RNase H$^-$ Reverse Transcriptase (Invitrogen, USA). After incubation of 2 μg total RNA with 0.5 μg oligo $(dT)_{12-18}$ primer at 70˚C for 10 min, the reaction was carried out in 5x first strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$), 10 mM DTT, and 0.5 mM dNTP containing a final volume of 20 μl mixture. Mixed contents of the tube were incubated at 42˚C for 2 min. SuperScript™ II RNase H$^-$ Reverse Transcriptase (Invitrogen) was then added and incubated for 50 min at 42˚C. PCR for *Mgl-1* was performed at 94˚C for 3 min, 55˚C for 30 sec, and 72˚C for 1 min for a total of 30 cycles, and *Sop1* cDNA was amplified at 94˚C for 3 min, 51˚C for 45 sec, and 72˚C for 45 sec for a total of 30 cycles. As an internal control, *ß-actin* (*Act1*) cDNA was also amplified using PCR primers at 94˚C for 3 min, 53˚C for 30 sec, and 72˚C for 45 sec for a total of 30 cycles.

Western blot analysis. As described previously (3), cells grown to OD_{600} 0.5 were harvested by spinning in a micro-centrifuge for 2 min. Harvested cells were resuspended in glass bead disruption buffer and mixed cell paste was washed. The proteins were separated by SDS-PAGE in 7.5% acrylamide gels and transferred to the Immobilon™-P Transfer Membrane (Millipore, USA) according to the manufacturer's protocol. The membranes were incubated with an anti-HA antibody (Roche Applied Science, Germany) and then probed with anti-mouse horseradish peroxidase-linked secondary antibodies (Amersham Pharmacia Biotech, England). Antibody detection was performed using the ECL labeling system (Amersham Pharmacia Biotech).

Complementation in yeast. The analysis for the restrictive temperature and salt tolerance of *Mgl-1* and its deletion mutants was performed in the *S. cerevisiae Lgl* mutants, *Sop1*Δ*Sop2*Δ. To analyze the cold-sensitive growth of *S. cerevisiae*, 10-fold dilutions of exponentially growing cells were spotted on YEPD agar plates and growth was assessed

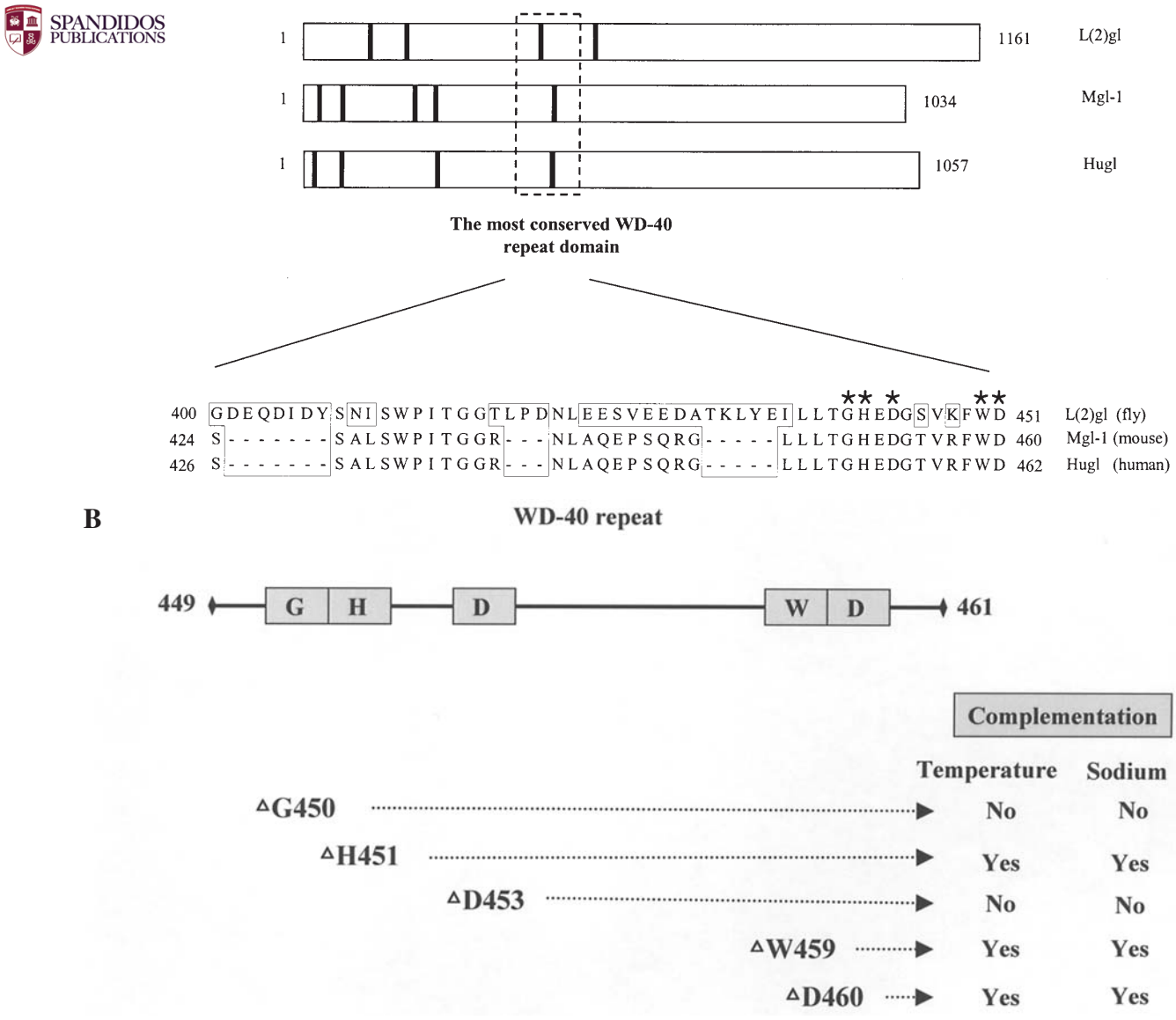

Figure 1. Sequence alignment of the WD-40 repeat motif for *Lgl* homologues and schematic representation of deletion mutation sites in the most conserved WD-40 repeat motif. (A) Representation of the WD-40 repeat motif (black bar) from the *Lgl* family members was mapped by a simple modular architecture research tool (SMART, http://smart.embl-heidelberg.de/). Sequence alignment of the *Lgl* family was done by the clustal method using the DNASTAR program. Three representative homologues are shown: L(2)gl (*Drosophila melanogaster*, gi157818), *Mgl-1* (*Mus musculus*, gi414350), and Hugl (*Homo sapiens*, gi784996). A box indicates different amino acid residues present in sequences. (B) To constitutively express the *Mgl-1* gene in *S. cerevisiae*, we subcloned the full length of *Mgl-1* into a yeast expression vector, pYX212 carrying the *TPI* promoter. The *Mgl-1* (ΔG450), *Mgl-1* (ΔH451), *Mgl-1* (ΔD453), *Mgl-1* (ΔW459), and *Mgl-1* (ΔD460) constructs were generated using the primers described in Table I. All constructs were confirmed by restriction analysis and sequencing. Five deletion mutation sites of the WD-40 repeat motif are schematically marked by asterisks. Results of the complementation test are shown by yes and no.

after incubation at 20˚C and 30˚C for 3-5 days. To determine tolerance to salt stress, 10-fold dilutions of an overnight culture diluted to OD_{600} 1.0 were spotted on YEPD agar plates containing 0.5 M NaCl. The plates were then incubated at 30˚C and growth was monitored.

Results and Discussion

Identification of *Lgl* family members in various organisms revealed a high similarity of amino acid sequence along with the presence of the WD-40 repeat motif in these proteins (Fig. 1A). It is becoming clear that the WD-40 repeat motif is involved in various cellular functions. In this study, we tried to analyze the cellular role of the WD-40 repeat motif using the complementation system in yeast. First of all, we generated deletion mutant forms of the most conserved WD-40 repeat motif for mouse *Mgl-1* (Fig. 1B) and transformed them into a mutant strain of yeast lacking *Sop1* and *Sop2*, the *Lgl* homologues in yeast. The expression of *Mgl-1* was confirmed by both RT-PCR and Western blot analyses (Fig. 2). A PCR product for *Sop1* cDNA was generated in wild-type strain *W303* (Fig. 2A, lane 1), but not in the mutant strain *Sop1*Δ*Sop2*Δ (Fig. 2A, lanes 2-9). PCR products for either wild-type *Mgl-1* or the deletion mutant forms of *Mgl-1* were confirmed in the absence of *Sop1* and *Sop2* (Fig. 2B, lanes 4-9). As an internal control, the expression of *Act1* was confirmed (Fig. 2C). In addition, the production of wild-type *Mgl-1* or the deletion mutant forms of HA-tagged *Mgl-1* protein was

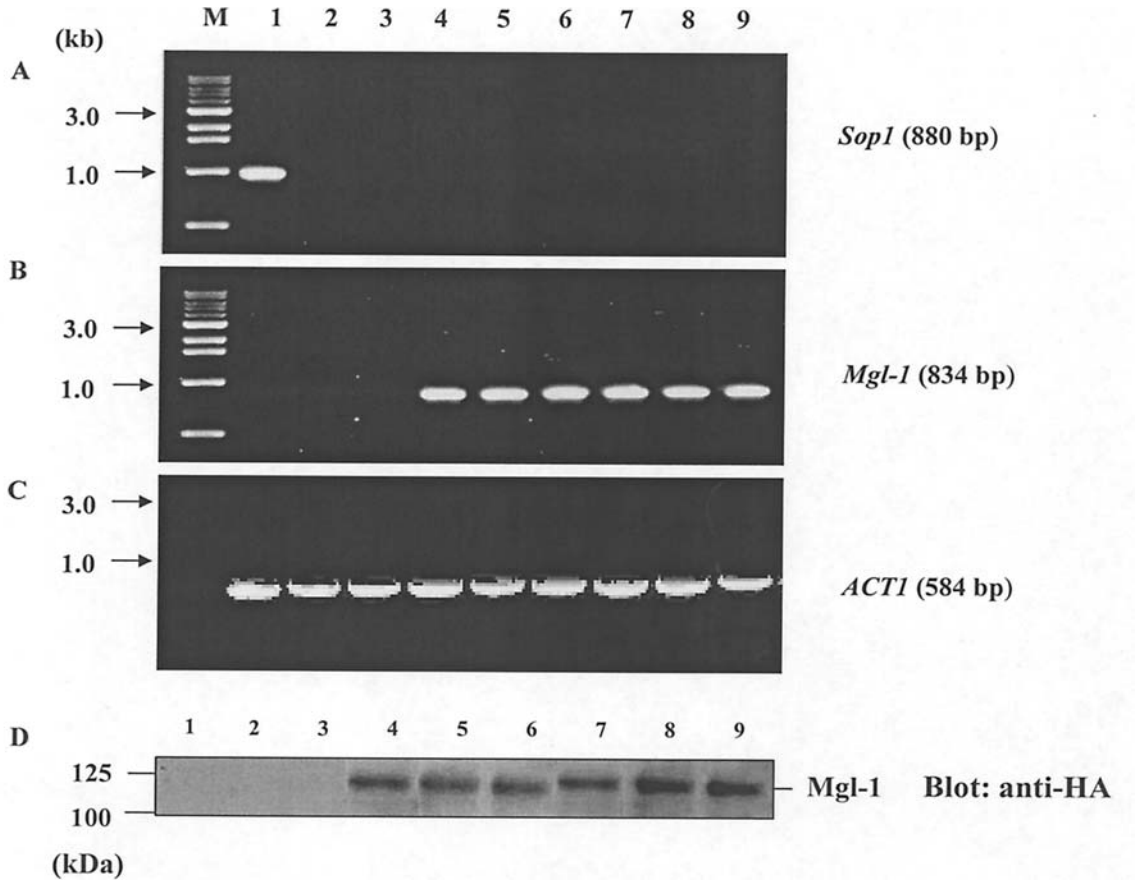

Figure 2. RT-PCR and immunoblot analyses showing the expression of the mouse *Mgl-1* and its mutants in *S. cerevisiae* transformants. *Mgl-1* transcripts are confirmed by RT-PCR analysis using specific primers for *Mgl-1* (Table I). The specific primers for *Sop1* and *Act1* were used as a control (Table I). (A) *Sop1* expression, (B) *Mgl-1* expression, (C) *Act1* expression. Lane M, 1 Kb DNA marker; lane 1, *W303* (wild-type strain); lane 2, *WKL-23* (*Sop1*Δ*Sop2*Δ mutant strain); lane 3, *WKL-23* + pYX212; lane 4, *WKL-23* + pYX212-*Mgl-1*; lane 5, *WKL-23* + pYX212-*Mgl-1* (ΔG450); lane 6, *WKL-23* + pYX212-*Mgl-1* (ΔH451); lane 7, *WKL-23* + pYX212-*Mgl-1* (ΔW459); lane 8, *WKL-23* + pYX212-*Mgl-1* (ΔD460); and lane 9, *WKL-23* + pYX212-*Mgl-1* (ΔD453). (D) *Mgl-1* and its mutant proteins expressed in *Sop1*Δ*Sop2*Δ double mutants were also confirmed by immunoblot analysis. The order of lanes is the same as for the RT-PCR analysis.

verified in yeast by Western blot analysis using an anti-HA antibody (Fig. 2D). HA-tagged *Mgl-1* was not expressed in wild-type strain *W303* or the double mutant strain *Sop1*Δ*Sop2*Δ, which were used as negative controls (Fig. 2D, lanes 1-2).

To examine whether the most conserved WD-40 repeat motif of *Mgl-1* is required for complementation in yeast in the absence of *Sop1* and *Sop2* genes, five deletion mutant forms of *Mgl-1* (ΔG450, ΔH451, ΔD453, ΔW459, and ΔD460) were transformed into the mutant yeast strain, which was grown either at the restrictive temperature (20˚C) or in YEPD medium containing high salt (0.5 M NaCl) conditions. The analysis for temperature-sensitivity revealed that the deletion mutant forms of *Mgl-1* at the conserved glycine residue (G) at position 450 and aspartic acid residue (D) at position 453 were not able to complement a double mutant strain of yeast *Sop1*Δ*Sop2*Δ (Fig. 3). The analysis for salt-sensitivity showed that the deletion mutant forms of *Mgl-1* at the conserved glycine residue (G) at position 450 and aspartic acid residue (D) at position 453 were not able to complement the double mutant strain of yeast *Sop1*Δ*Sop2*Δ (Fig. 4), as shown with temperature-sensitivity analysis. Since a number of proteins including *Mgl-1* are involved in the formation of the cytoskeletal complex (5), it is possible that structural modification by mutations may lead to the aberration of cytoskeletal complex resulting in no complementation.

Previously, we carried out a similar complementation analysis with 5 point mutant forms of *Mgl-1* (G450R, H451Q, W459R, D453N and D460N) in yeast (19). The results also showed that point mutant forms of *Mgl-1* at the conserved glycine at position 450 and aspartic acid at position 453 in the most conserved WD-40 repeat motif were not able to complement, indicating that these amino acids are critical for regulating salt tolerance and temperature-sensitivity in yeast. It has been suggested that the side chain oxygens of the conserved aspartic acid residue (D) in the WD-40 repeat motif form hydrogen bonds with main chain nitrogen atoms both in the tight turn and in the loop connecting the first and second strand of the same sequence repeat, which is the loop between the two adjacent blades (20,21). This hydrogen bonding arrangement stabilizes and effectively couples the tight turn of one ß sheet to the outer strand of the previous ß sheet (20), suggesting a major role in protein-protein interaction. Therefore, our finding strongly supports the cellular role of WD-40 repeat motif in *Lgl* family members.

Over 30 functional subfamilies among the WD-40 repeat-containing proteins observed in the genomes have been reported (22), suggesting that these proteins play an important role in a variety of cellular functions. These subfamilies can

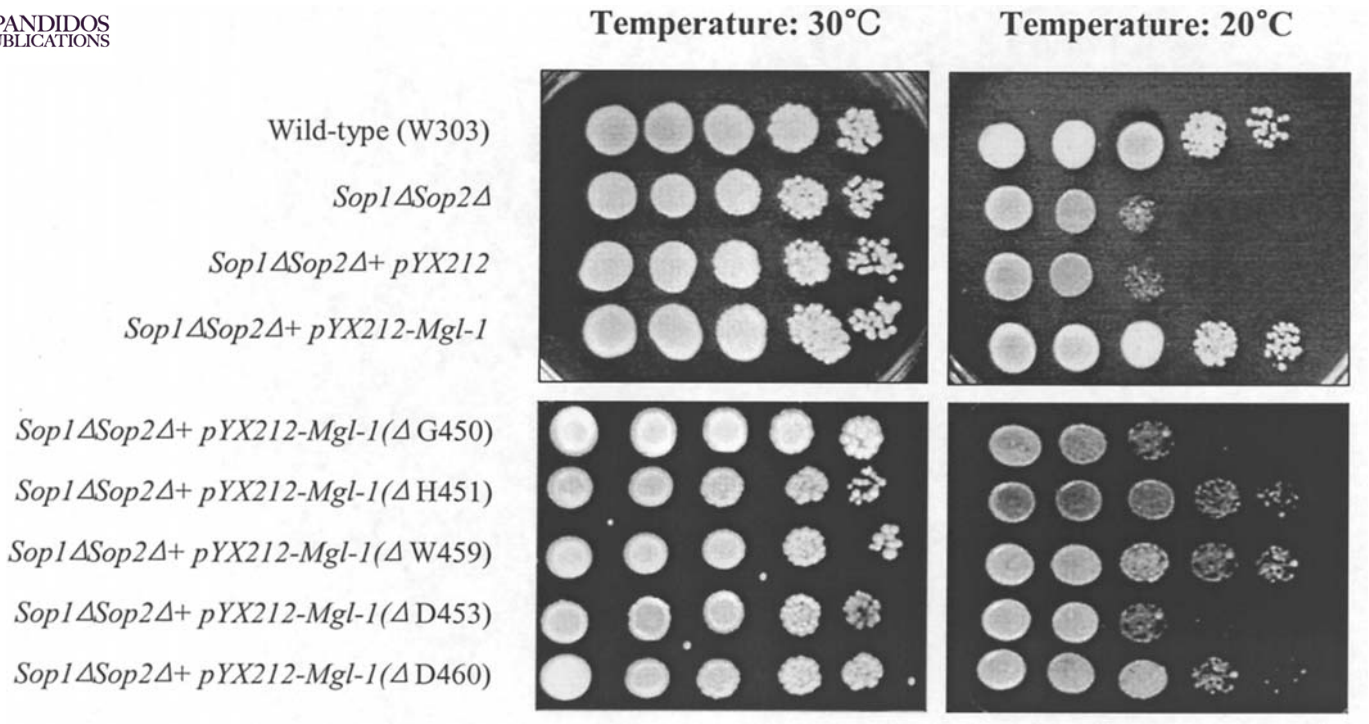

Figure 3. Complementation of temperature-sensitivity of *Sop1*Δ*Sop2*Δ double mutants. The *Sop1*Δ*Sop*Δ mutants were transformed with either the *Mgl-1* cDNA or each of *Mgl-1* mutant cDNAs inserted in the multicopy pYX212 plasmid. Cells were grown overnight in YEPD medium, adjusted to OD_{600} 1.0 and serial 10-fold dilutions were spotted on YEPD plates. They were then incubated at 20˚C or 30˚C for 3-5 days.

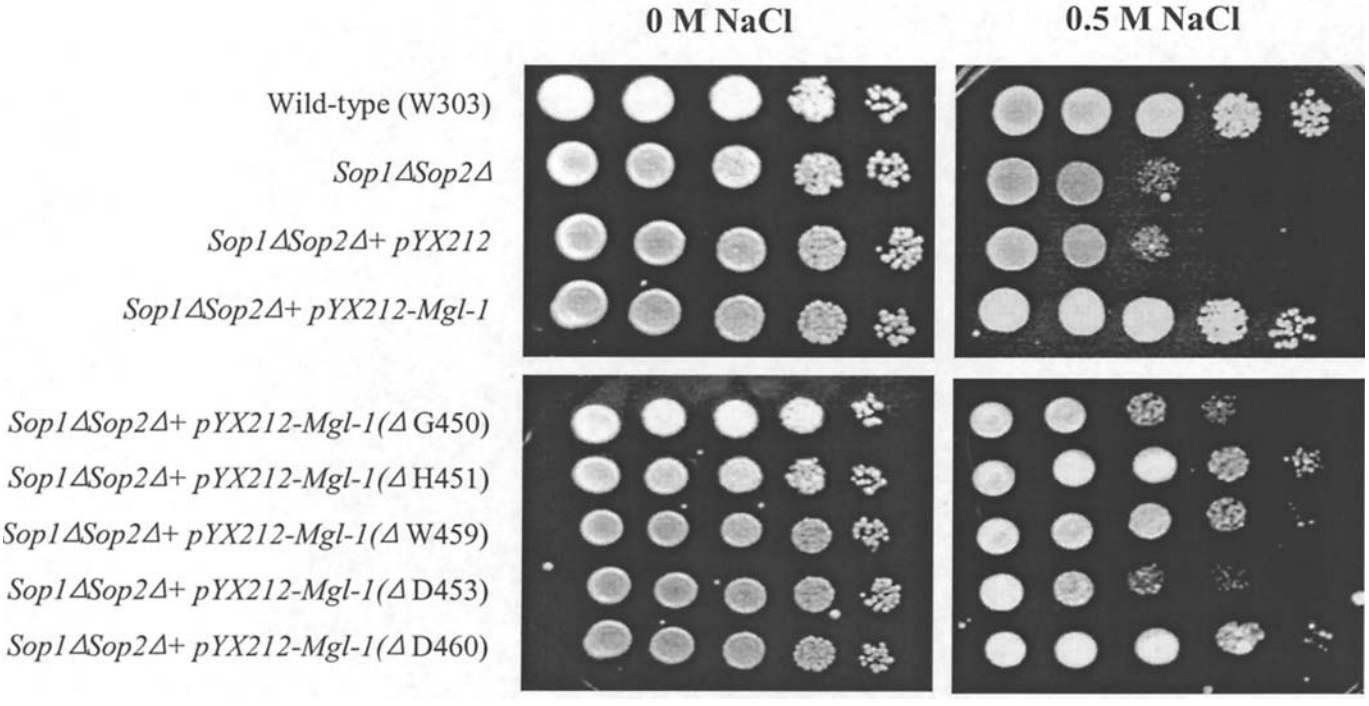

Figure 4. Complementation of salt tolerance of *Sop1*Δ*Sop2*Δ double mutants. The *Sop1*Δ*Sop*Δ mutants were transformed with either the *Mgl-1* cDNA or each of *Mgl-1* mutant cDNAs inserted in the multicopy pYX212 plasmid. Cells were grown overnight in YEPD medium, adjusted to OD_{600} 1.0 and serial 10-fold dilutions were spotted on YEPD plates containing 0 M or 0.5 M NaCl. They were then incubated at 30˚C for 3-5 days.

be classified into signal transduction, RNA synthesis/processing, chromatin assembly, vesicular assembly, cell cycle control, and apoptosis (17). Therefore, detailed structural and cellular analyses for the role of the WD-40 repeat motif in the *Lgl* family will provide some insights into the mechanism of tumor development along with cellular polarity and cytoskeletal complex formation.

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