

# The *Saccharomyces cerevisiae* WRN homolog Sgs1p participates in telomere maintenance in cells lacking telomerase

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**Werner syndrome (WS) is marked by early onset of features resembling aging, and is caused by loss of the RecQ family DNA helicase WRN. Precisely how loss of WRN leads to the phenotypes of WS is unknown. Cultured WS fibroblasts shorten their telomeres at an increased rate per population doubling and the premature senescence this loss induces can be bypassed by telomerase. Here we show that WRN co-localizes with telomeric factors in telomerase-independent immortalized human cells, and further that the budding yeast RecQ family helicase Sgs1p influences telomere metabolism in yeast cells lacking telomerase. Telomerase-deficient *sgs1* mutants show increased rates of growth arrest in the G<sub>2</sub>/M phase of the cell cycle as telomeres shorten. In addition, telomerase-deficient *sgs1* mutants have a defect in their ability to generate survivors of senescence that amplify telomeric TG<sub>1-3</sub> repeats, and *SGS1* functions in parallel with the recombination gene *RAD51* to generate survivors. Our findings indicate that Sgs1p and WRN function in telomere maintenance, and suggest that telomere defects contribute to the pathogenesis of WS and perhaps other RecQ helicase diseases.**

**Keywords:** ALT/replicative senescence/*SGS1*/telomeres/Werner syndrome

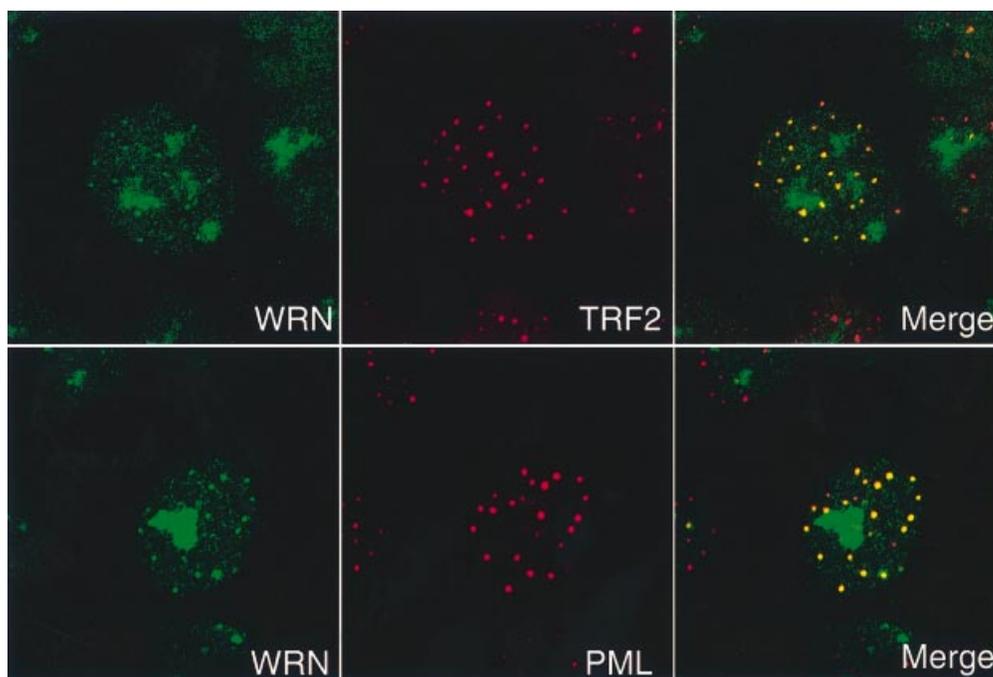
## Introduction

Three human diseases are caused by loss of RecQ family DNA helicases: Werner syndrome (WS), Rothmund-Thompson syndrome (RTS) and Bloom syndrome (BS) (Ellis *et al.*, 1995; Yu *et al.*, 1996; Kitao *et al.*, 1999). WS, RTS and, to a small degree, BS are marked by the premature onset of features resembling aging (Epstein *et al.*, 1966; German, 1995; Lindor *et al.*, 2000), arguing that this class of proteins has roles in preventing the accumulation of genomic lesions that contribute to phenotypes of aging. Individuals with WS are essentially

normal as children, but as young adults develop premature graying and loss of hair, thinning of the dermis and loss of subcutaneous fat, bilateral ocular cataracts, and forms of osteoporosis and arteriosclerosis (Epstein *et al.*, 1966). They also have an increased incidence of cancer, in particular sarcomas, a feature shared with RTS (Lindor *et al.*, 2000). Several abnormalities in WS cells have been described, including elevated levels of DNA deletions and chromosome translocations, and sensitivity to 4-nitroquinoline-1-oxide and the topoisomerase I poison camptothecin (reviewed by Shen and Loeb, 2000). Which, if any, of these defects contributes to the gross pathology of WS is unknown.

Replicative senescence refers to the finite replicative potential of many cultured human cell types (Hayflick, 1980). Most somatic human cells, including primary fibroblasts, lack telomerase activity and shorten their telomeres with cell division (Harley *et al.*, 1990). The shortening of telomeres appears to be a cause of replicative senescence because transgenic expression of telomerase can bypass senescence in several cell types (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998; Yang *et al.*, 1999). Cultured WS fibroblasts senesce prematurely, and shorten their telomeres at a faster rate per population doubling (Schulz *et al.*, 1996); studies of replicative senescence in RTS and BS have not been reported. As in wild-type (wt) fibroblasts, the senescence of WS and BS fibroblasts can be bypassed by expression of telomerase (Ouellette *et al.*, 2000; Wyllie *et al.*, 2000; our unpublished observations), arguing that telomeric events, rather than other cellular defects, lead to senescence in these mutant cells.

Similar to primary human fibroblasts, yeast cells in which telomerase has been genetically inactivated lose telomeric repeats and senesce with cell division (Lundblad and Szostak, 1989). For example, *Saccharomyces cerevisiae* cells lacking the telomerase RNA component *TLC1* shorten telomeres and lose viability, resulting in the death of most cells in the population (Singer and Gottschling, 1994). The terminal phenotype of senescing yeast cells has not been characterized. Rare cells escape senescence and these survivors maintain their telomeres without telomerase (Lundblad and Blackburn, 1993). This maintenance depends on the recombination gene *RAD52* and either *RAD50* or *RAD51* (Lundblad and Blackburn, 1993; Le *et al.*, 1999; Teng and Zakian, 1999), and so appears to involve recombination among telomeric DNA repeats (reviewed by Kass-Eisler and Greider, 2000). Two different types of survivors arise: type I have a repeat consisting of subtelomeric Y' elements and short telomeric TG<sub>1-3</sub> repeats amplified at their chromosome termini, while type II survivors have little amplification of Y' elements, but instead have long TG<sub>1-3</sub> repeats extending up to several kilobase pairs (kbp) (Lundblad and Blackburn, 1993; Teng and Zakian, 1999).



**Fig. 1.** Co-localization of WRN with telomeric factors in human ALT fibroblasts. Cultured WI38 75.1 fibroblasts were examined by indirect immunofluorescence and confocal microscopy with antibodies to WRN, TRF2 and PML, as indicated. The cell in the center of the field showing co-localization of WRN with TRF2 and PML is representative of the 5% of cells in a WI38 75.1 culture that possess AA-PML bodies.

Telomerase-independent telomere maintenance mechanisms also occur in human cells. Primary human cells may be induced to bypass senescence by oncoproteins, such as SV40 large T antigen. Such cells shorten their telomeres to a point referred to as crisis, where the shortened telomeres lead to genome instability and apoptosis. Rare cells survive crisis and maintain their telomeres either by expression of telomerase or by a telomerase-independent mechanism (or mechanisms) termed ALT (alternative lengthening of telomeres; Bryan *et al.*, 1997). ALT cells are immortalized and are characterized by telomeres of heterogeneous size that extend beyond 50 kbp in length (reviewed in Colgin and Reddel, 1999). ALT cells are also distinguished by nuclear structures, referred to as ALT-associated promyelocytic leukemia (AA-PML) bodies, which contain telomeric repeat DNA, the telomere repeat binding proteins TRF1 and TRF2, and the PML protein (Yeager *et al.*, 1999). ALT is believed to involve recombination among telomeric DNA repeats, based largely on analogy with yeast survivors of senescence. Observations supporting similarities between yeast survivor pathways and human ALT include: (i) the long and heterogeneous telomeric repeats of type II survivors and of human ALT cells; and (ii) the requirements for *RAD50*, *RAD51* and *RAD52* in yeast survivor pathways, and the presence of human *RAD50*, *RAD51* and *RAD52* proteins in AA-PML bodies (Yeager *et al.*, 1999; Zhu *et al.*, 2000). Approximately 5% of human tumors use an ALT mechanism to maintain telomeres (Bryan *et al.*, 1997; Colgin and Reddel, 1999).

*Saccharomyces cerevisiae* has a single RecQ family member, *SGS1* (Gangloff *et al.*, 1994). *Sgs1* mutants display elevated levels of recombination, particularly among repeated DNA sequences including the ribosomal DNA and subtelomeric Y' elements (Gangloff *et al.*, 1994;

Watt *et al.*, 1996). When combined with mutation of another helicase, *SRS2*, *sgs1* mutation causes extremely poor growth. The poor growth can be bypassed by *rad51* mutation, indicating that *SGS1* is important for preventing deleterious recombination events (Gangloff *et al.*, 2000). WRN and BLM can each complement certain *sgs1* recombination phenotypes, demonstrating conservation of function among the family of helicases (Yamagata *et al.*, 1998).

Here we demonstrate localization of WRN with telomeric factors in ALT cells, a finding that prompted us to study telomeric functions of *SGS1*. In cells lacking telomerase, *sgs1* mutants senesce prematurely and shorten their telomeres rapidly. We demonstrate for the first time that senescing yeast cells arrest at a specific phase in the cell cycle, G<sub>2</sub>/M. Senescing *sgs1* mutant cells show higher rates of growth arrest at any given telomere length, a phenotype which is not observed in telomerase-positive *sgs1* cells. We also show that *SGS1* functions in parallel with *RAD51* in the generation of survivors of senescence. The findings support the hypothesis that telomere defects contribute to the pathogenesis of WS and perhaps RTS and BS.

## Results

### **Localization of WRN with telomeric factors in human ALT cells**

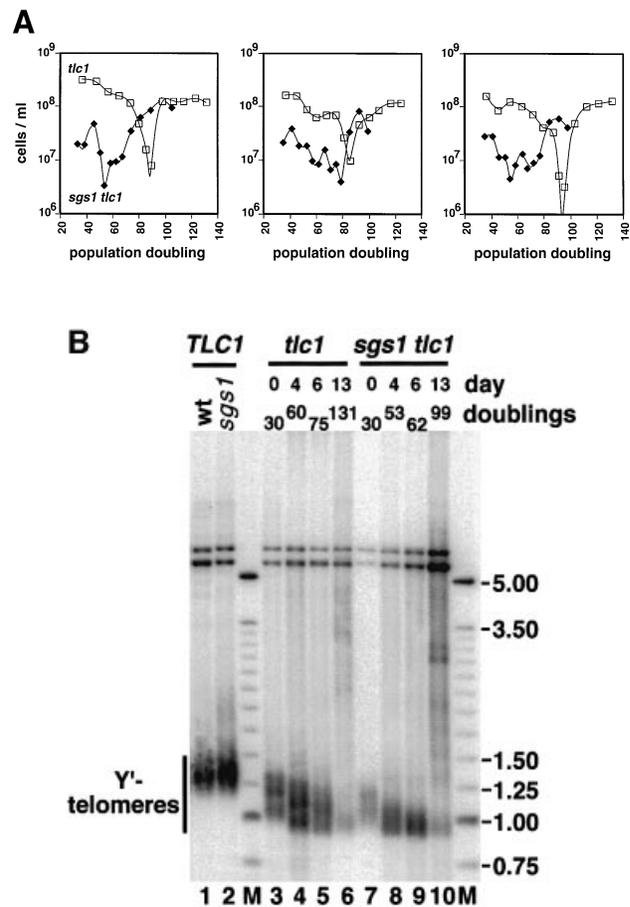
WRN localizes predominantly in the nucleolus in many cell types (Marciniak *et al.*, 1998). However, indirect immunofluorescence of WRN in immortalized human cells that lack telomerase, ALT cells (Bryan *et al.*, 1997), revealed that in addition to nucleolar localization, WRN co-localizes in nuclear foci with the telomere repeat binding factors TRF2 (Figure 1) and TRF1 (data not

shown). ALT cells are characterized by long and heterogeneous telomeres and by nuclear structures, dubbed AA-PML, containing telomeric repeat DNA, telomere repeat binding factors and several proteins involved in DNA recombination; these bodies are typically present in 5% of cycling interphase cells (Yeager *et al.*, 1999). WRN localizes to such bodies, as assessed by co-staining known AA-PML body proteins (Yeager *et al.*, 1999; Zhu *et al.*, 2000), including PML, hRAD52 and NBS1 (Figure 1 and data not shown). WRN co-localization with TRF1 and TRF2 was observed in all six ALT cell lines examined. These included both SV40 T-antigen-transformed cell lines (WI38 75.1, VA13, LM216J and AG07066) and spontaneously occurring tumor cell lines (Saos2 and U2OS). We did not observe co-localization of WRN with PML or telomeric factors in primary fibroblast cell lines (e.g. BJ, and WI38) or in telomerase-positive cell lines (e.g. HeLa, and hTERT-immortalized BJ fibroblasts). Our inability to detect WRN at telomeres in primary or telomerase-positive cells may reflect a specific association between WRN and ALT-maintained telomeres. Alternatively, these findings may be a consequence of the limited sensitivity of immunofluorescence, because many proteins present at telomeres in both ALT and primary cells are detected more readily in ALT cells (e.g. NBS1; Zhu *et al.*, 2000). Nonetheless, the co-localization of WRN with telomere components under at least some conditions suggests that WRN could have direct effects at telomeres.

#### Premature senescence of yeast *sgs1* mutants

The above findings prompted us to investigate a possible role for the yeast WRN homolog *SGS1* in telomere metabolism. *SGS1* has been reported to have no effect on steady-state telomere length, although *sgs1* mutants do have elevated levels of recombination among subtelomeric *Y'* elements (Watt *et al.*, 1996). Yeast have constitutive telomerase activity, but genetic inactivation of components of the yeast telomerase complex causes telomeres to shorten with each cell division and results in the eventual senescence of the clone, similar to the telomere shortening and senescence of cultured human primary fibroblasts (Lundblad and Szostak, 1989; Singer and Gottschling, 1994).

To investigate functions of *SGS1* in senescing yeast cells, we sporulated diploids heterozygous for deletion mutations in *SGS1* and the telomerase RNA *TLC1* (Singer and Gottschling, 1994). The *sgs1 tlc1* double mutant segregants senesced more rapidly than *tlc1* mutants in liquid culture (Figure 2A; see Materials and methods) and in serial streaks on plates (not shown). Telomere length in the senescing cultures was assessed by probing *XhoI*-digested DNA with a fragment of the subtelomeric *Y'* elements present on most chromosome ends (e.g. Figure 2B). As reported (Watt *et al.*, 1996), *sgs1* mutants had telomere lengths similar to wt (Figure 2B, lanes 1 and 2 and data not shown), although analysis of 13 pairs of *SGS1* and *sgs1* segregants did indicate a slightly greater length in the *sgs1* mutants (mean lengths and standard deviations of *Y'*-containing terminal restriction fragments:  $1301 \pm 35$  versus  $1324 \pm 30$  bp,  $P < 0.1$ ). To measure initial telomere shortening rates, isolated segregants were grown in liquid culture until sufficient cells were obtained



**Fig. 2.** Senescence of *tlc1* and *sgs1 tlc1* cells. (A) Senescence in liquid culture. Values are from three independent pairs of *tlc1* (open squares) and *sgs1 tlc1* (closed diamonds) mutant cultures for the first 13 days of growth in liquid. Control wt and *sgs1* cultures grown in parallel gave mean levels of  $1.9 \times 10^8$  and  $1.5 \times 10^8$  cells/ml. Similar results were obtained for the first 3 days of liquid culture comparing 16 other spore pairs from an independent cross. (B) *Y'*-containing telomeres were visualized by digesting genomic DNA with *XhoI* and probing with *Y'* sequences distal to the cut site. Lanes 1 and 2 are wt and *sgs1* mutant cells, and lanes 3–6 and 7–10 are from senescing *tlc1* and *sgs1 tlc1* mutants, respectively. Lanes 3 and 6 are from reference patches of segregants (day 0) and other lanes are from liquid culture. Days of growth in liquid, population doubling levels and size markers (M, in kbp) are indicated.

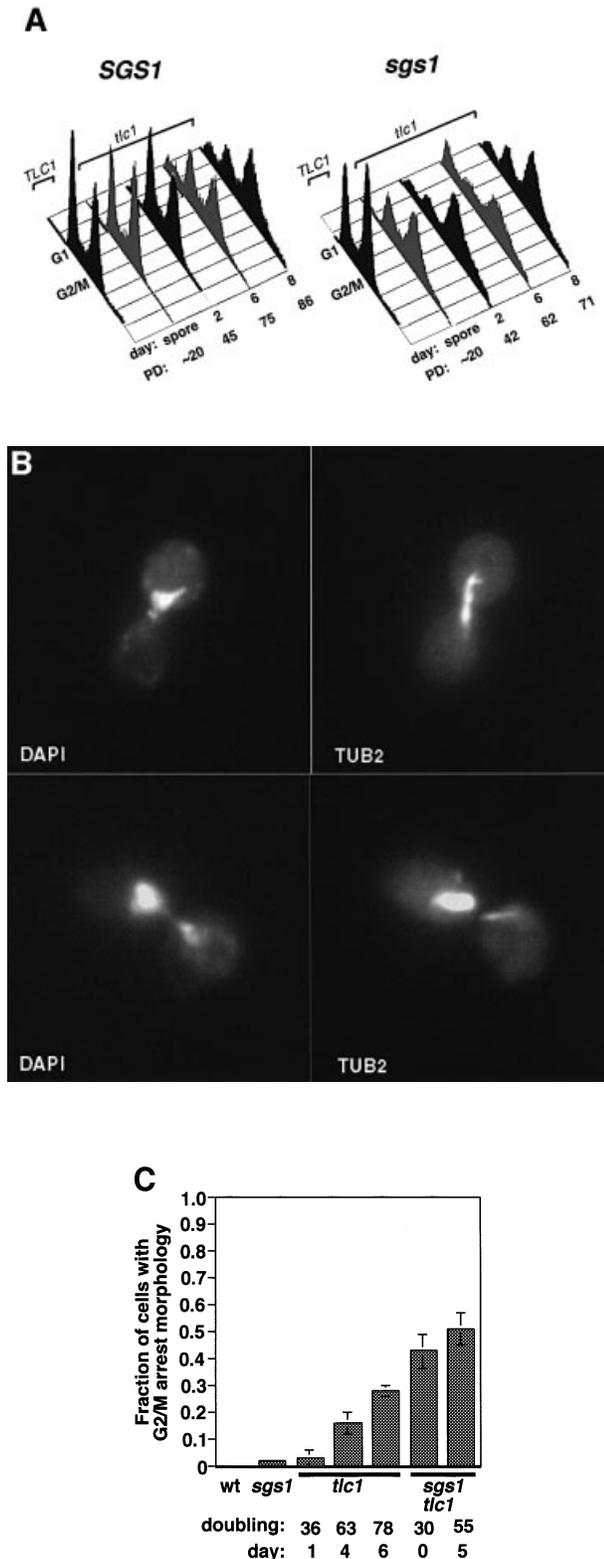
for Southern analysis of *Y'*-containing telomere lengths, and the number of population doublings was calculated (see Materials and methods). Comparing senescing segregants to the wt segregant within each tetrad yielded mean rates and standard deviations of  $5.7 \pm 1.0$  and  $7.2 \pm 1.7$  bp per population doubling for the *tlc1* single and *sgs1 tlc1* double mutants, respectively ( $N = 13$  pairs,  $P < 0.01$ ). Comparing *tlc1* to wt and *sgs1 tlc1* to *sgs1* segregants within each tetrad yielded shortening rates of  $5.6 \pm 1.0$  and  $8.0 \pm 1.2$  bp per population doubling ( $N = 12$  pairs,  $P < 0.001$ ). Thus, like senescing cultured WS fibroblasts, *sgs1 tlc1* mutants shorten telomeres at an increased rate per population doubling.

#### *Sgs1* mutants have a hypersensitive arrest phenotype in the absence of telomerase

The faster telomere shortening in *sgs1 tlc1* cultures could result from effects of Sgs1p on the rate of telomere

shortening in dividing cells or from an increased tendency of *sgs1* mutants to cease cell division as telomeres shorten. In the latter case, viable cells must undergo additional divisions to compensate for arrested cells, thereby further shortening their telomeres and clonal lifespan. We thus examined the phenotypes of senescing cells, using flow cytometry and microscopy. *tlc1* mutants accumulated in

the G<sub>2</sub>/M phase of the cell cycle as they approached senescence (Figure 3A). The accumulation of cells in the G<sub>2</sub>/M compartment is accompanied by an increase in cells with <1N (and perhaps >2N) DNA content. These changes might reflect debris from dying cells or perhaps chromosome fragmentation or aneuploidy in the senescing cells. The progressive G<sub>2</sub>/M accumulation also occurred in the *sgs1 tlc1* mutants, but was more dramatic and occurred at earlier generations and longer telomere lengths. For example, the cell cycle pattern for generation 20 *sgs1 tlc1* cells is not seen until generation 75 for *tlc1* cells, yet the telomeres of generation 30 *sgs1 tlc1* cells are clearly longer than those of generation 75 *tlc1* cells (Figure 2B, lanes 5 and 7). Fluorescence microscopy of cells stained with 4',6-diamidino-2-phenylindole (DAPI) and anti-tubulin antibodies to visualize DNA and the nuclear spindle revealed an accumulation of large budded cells, many with nuclei wedged in bud necks and with short spindles (Figure 3B and data not shown), characteristic of G<sub>2</sub>/M arrest (Pinto and Winston, 2000). The frequency of such cells paralleled the changes observed by flow cytometry, and the *sgs1 tlc1* mutants again arrested with this morphology more frequently at longer telomere lengths (Figure 3C). Cells with the G<sub>2</sub>/M phenotype might have permanently arrested or might have only paused in their cell cycle progression. To distinguish between these possibilities, we tested cells from senescing liquid cultures for their ability to divide after growth for 36 h on YPD plates. Cells and microcolonies were observed by microscopy, and isolated large single- or double-budded cells were considered to have arrested permanently. After the first day of growth in liquid media, 28% of *sgs1 tlc1* cells failed to divide, compared with only 1, 5 and 22% of the *tlc1* cells on the first, fourth and seventh days, respectively. Under these conditions, >98% of control wt and *sgs1* cells formed colonies. In conclusion, a greater fraction of *sgs1 tlc1* mutant cells permanently exit the cell cycle (and presumably die) at all telomere lengths as the culture senesces. This G<sub>2</sub>/M arrest occurs in only a small fraction of *sgs1* cells (Figure 3C), indicating that the G<sub>2</sub>/M arrest in *tlc1* and *sgs1 tlc1* cells is due to a telomere-related defect. This might reflect a role for Sgs1p helicase activity in avoiding or resolving deleterious recombination events at telomeres.



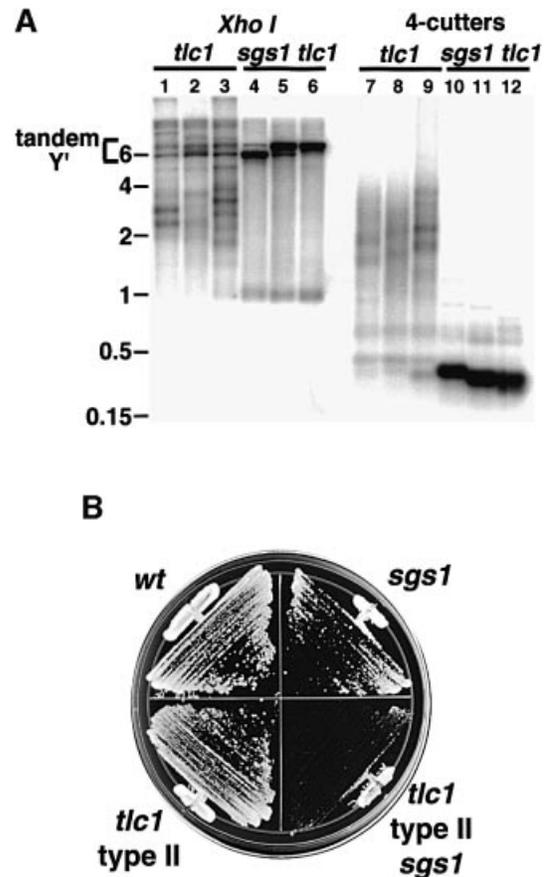
**Fig. 3.** Arrest phenotypes of senescing *tlc1* and *sgs1 tlc1* cells. (A) The first cell cycle profile on each graph is from wt or *sgs1* cells, which differed in the fraction of cells in G<sub>2</sub>/M (44 and 55%, respectively). The later profiles are senescing *tlc1* or *sgs1 tlc1* cells from one spore pair, taken from segregant colonies or on the indicated day and population doubling (PD) of liquid culture (same cultures as used for Figure 2B). Similar results were obtained for two other spore pairs. Note that the day 8 *sgs1 tlc1* culture contains ALT survivors. (B) Two examples of typical large-budded *sgs1 tlc1* cells at day 5 of liquid culture, apparently arrested in G<sub>2</sub>/M, stained with DAPI and anti-TUB2 tubulin antibodies. Arrested *tlc1* cells had the same phenotype as *sgs1 tlc1* cells (not shown). (C) Quantitation of the fraction of cells with large buds, short nuclear spindles and nuclei near or in the bud neck. Mean and standard deviations for three cultures of each type are shown; at least 100 cells were counted for each sample. Genotypes and population doublings and days in liquid culture are indicated. The mean telomere lengths and standard deviations for *sgs1 tlc1* on day 0, and for *tlc1* on day 4 and 6 were 1139 ± 44, 1142 ± 15 and 1096 ± 14 bp, respectively.

### Role of *SGS1* in the type II survivor pathway

Although most yeast cells lacking telomerase cease dividing because of shortening telomeres, a small fraction of cells escape senescence and maintain their telomeres by recombination-dependent mechanisms (Lundblad and Blackburn, 1993; Teng and Zakian, 1999). The most frequent type of survivor (type I) maintains telomeres by amplifying a unit consisting of a subtelomeric *Y'* element and a short stretch of telomeric  $TG_{1-3}$  DNA, and has a variable growth rate. With continued cell division, type I cells may convert to faster growing type II survivors, which maintain telomeres via amplification of long and heterogeneous terminal  $TG_{1-3}$  repeats. Conversion from type I to type II is seen most readily in liquid culture where the more rapidly growing cells overtake the population (Teng and Zakian, 1999). *sgs1 tlc1* mutants generated survivors with efficiency similar to *tlc1* mutants, although the emergence of survivors was preceded by a more prolonged period of poor growth in the double mutants (Figure 2A and data not shown). To test whether this period of poor growth of *sgs1 tlc1* cells might be associated with a qualitative defect in the generation of survivors, we examined the telomeres of survivors by Southern analysis. Genomic DNA was digested with *XhoI*, or a combination of endonucleases with four-base recognition sequences that do not cut  $TG_{1-3}$  repeats, and probed with poly(dG)-(dT); type I cells yield discrete bands corresponding to amplified *Y'* DNA, and type II cells yield more heterogeneous patterns (Teng and Zakian, 1999). Twelve *tlc1* and nine *sgs1 tlc1* survivor colonies each generated from independent spore segregants were grown for 120 generations in liquid culture and monitored for the emergence of type II survivors. Ten of the *tlc1* cultures adopted type II character by 10 generations of growth, one by 20 generations, and one maintained a mixed type I/II phenotype for 120 generations (Figure 4A, lanes 1–3 and 7–9 and data not shown). In contrast, all of the *tlc1 sgs1* cultures maintained a clear type I character for at least 120 generations (Figure 4A, lanes 4–6 and 10–12 and data not shown), indicating that *SGS1* is important for the transition from type I to II. When *SGS1* was deleted from established type II survivors, the double mutant survivors grew much more poorly than *sgs1* cells or *tlc1* survivors (Figure 4B). The poor growth of *sgs1 tlc1* type II survivors might be due to difficulties in the replication or stabilization of  $TG_{1-3}$  repeats, and may explain why these cells do not accumulate in liquid culture.

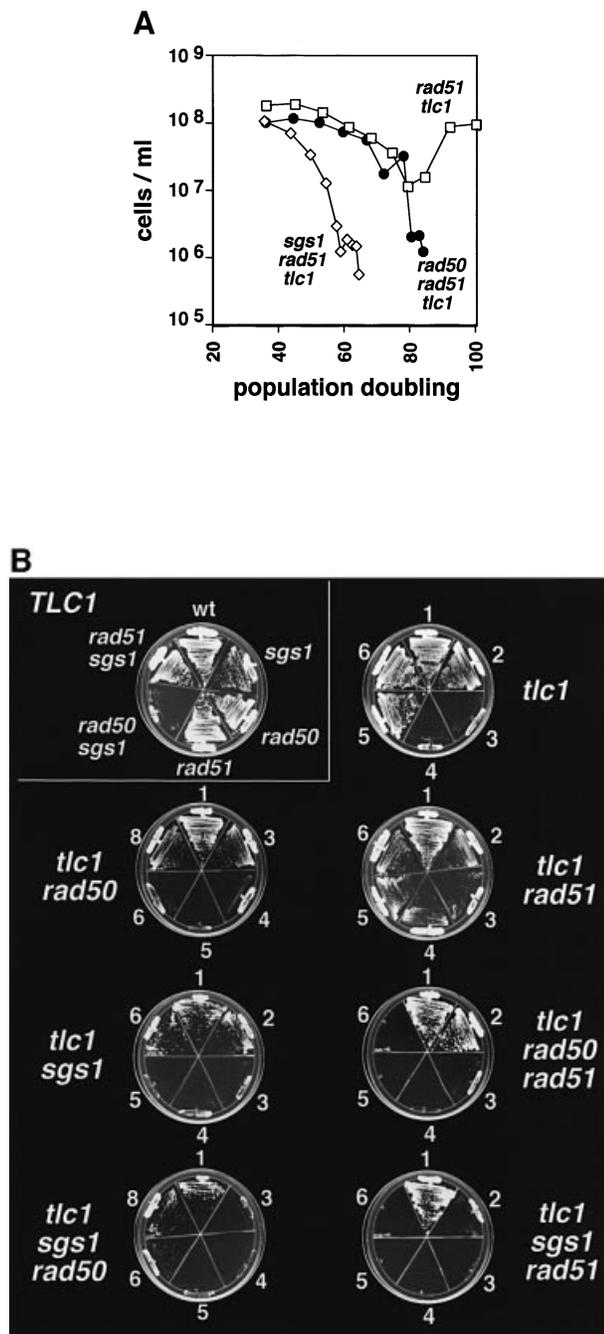
### *SGS1* is important for the *RAD51*-independent survivor pathway

There are at least two recombination pathways by which *tlc1* survivors can arise, involving either *RAD50* or *RAD51* (Le *et al.*, 1999). We addressed whether *Sgs1p* was a component of either pathway by which the *RAD* genes. Diploids triply heterozygous for *tlc1*, *sgs1* and *rad50* or *rad51* deletions were sporulated and the segregants were carried through senescence in liquid (Figure 5A) or on plates (Figure 5B and data not shown). *rad50 rad51 tlc1* mutants were also generated as controls. As expected, each of 13 *tlc1* and nine *sgs1 tlc1* mutants generated survivors on plates (Figure 5B and data not shown) just as they had in liquid culture (Figure 2A). *sgs1 rad50 tlc1* cells, although slow growing, also generated survivors



**Fig. 4.** Dependence of senescence survivor phenotypes on *SGS1*. (A) Examples of telomere structures of *tlc1* survivors, three *tlc1* (lanes 1–3 and 7–9) and three *sgs1 tlc1* (lanes 4–6 and 10–12). Newly emerged and independent survivor colonies from senescence on plates were grown for an additional 120 generations in liquid culture. Genomic DNA was digested with *XhoI*, which enables visualization of tandemly amplified *Y'* elements (type I pattern), or with *AluI*, *HaeIII*, *HinfI* and *MspI*, which digest genomic DNA into small fragments but leave amplified  $TG_{1-3}$  sequences intact (type II pattern) (Teng and Zakian, 1999). Blots of digested DNA were probed with poly(dG)-(dT) to detect all telomeric tracts, as well as *Y'* sequences, which have short interspersed dG-dT DNA (Lundblad and Szostak, 1989; Le *et al.*, 1999). Note that the two *Y'* bands correspond to the two different size classes of *Y'* elements: *Y'S* and *Y'L*. (B) Growth after 42 h on rich media of wt, *sgs1*, a type II survivor and the same type II survivor from which *SGS1* has been deleted.

efficiently (Figure 5B and data not shown). In contrast, *sgs1 rad51 tlc1* mutants, like *rad50 rad51 tlc1* mutants, grew well initially but failed to generate survivors in liquid culture (Figure 5A). In 17 of 18 trials on plates, no *sgs1 rad51 tlc1* survivors were observed (Figure 5B and data not shown). In the 18th trial, as well as in nine of 17 trials for *rad50 rad51 tlc1* mutants, survivors appeared. In this same *rad50 rad51 tlc1* strain, survivors were not reported previously (Le *et al.*, 1999). The larger number of cells used for each passage in our plate assays ( $>10^7$ ), compared with  $2 \times 10^6$  for liquid assays, may have allowed for the selection of rare bypass suppressor mutants. We conclude that *SGS1* functions in parallel with *RAD51* in the generation of survivors, and plays a role at least as important as *RAD51* in the generation of survivors that emerge in the absence of *RAD51*.



**Fig. 5.** *SGS1* dependence of the emergence of survivors in *tlc1* cells lacking *RAD50* or *RAD51*. (A) Senescence of *rad51 tlc1*, *rad50 rad51 tlc1* and *sgs1 rad51 tlc1* mutants in liquid culture, using three, two and five independent spores, respectively. The mean results for days 1–10 in liquid culture are shown. (B) Examples of senescing cells during growth on solid medium. For comparison, the upper left plate shows growth of *TLC1* cells of the indicated genotypes after 42 h of growth. Clockwise from the top, the genotypes are wt, *sgs1*, *rad50*, *rad51*, *sgs1 rad50* and *sgs1 rad51*. The remaining plates illustrate serial restreaks of senescing cells of the indicated genotypes (restreak number is indicated; 1 is the first restreak from the reference patch made from spore colonies). Cultures were first carried through senescence on separate plates and the time course was then reconstructed on the plates shown. The genotypes are *tlc1*, *tlc1 rad50*, *tlc1 rad51*, *tlc1 sgs1*, *tlc1 rad50 rad51*, *tlc1 sgs1 rad50* and *tlc1 sgs1 rad51*, moving top to bottom and left to right. Plates were incubated for 54 h, except for *tlc1* and *tlc1 rad51*, which were incubated for 42 h, and *tlc1 sgs1 rad50*, which was incubated for 67 h to adjust for different growth rates. *sgs1 rad51* double mutants cultured in parallel did not senesce (not shown).

## Discussion

We have observed defects in *sgs1* mutant cells lacking telomerase, both during the senescence of the cells and in survivors of senescence. The central defect in senescing *sgs1* cells is a greater tendency to arrest growth as telomeres shorten. There are three related, and non-exclusive, interpretations of this defect. One possibility is that loss of telomerase or telomere shortening causes structural changes that induce recombination or the formation of G-quadruplex complexes (see below) between telomeres. If such events cannot be resolved efficiently in the absence of the Sgs1p helicase, G<sub>2</sub>/M arrest could result. Secondly, if *SGS1* plays a role in an intra-S checkpoint (Frei and Gasser, 2000), in *sgs1* mutants telomere lesions may persist through S phase and later trigger a G<sub>2</sub>/M arrest. A third possibility is that *SGS1* is normally involved in a telomerase-independent repair pathway, perhaps mechanistically related to its role in the survivor pathway (see below). In this model, a telomere suddenly and critically shortened by damage at a point in the cell cycle when telomerase is not active [telomerase appears only to lengthen telomeres in late S phase (Marcand *et al.*, 2000)] might be repaired by an *SGS1*-dependent pathway. Our data indicate that the increased rate of telomere shortening in senescing *sgs1 tlc1* populations is most simply explained by a more rapidly diminishing fraction of dividing cells, although an additional direct effect on the rate of shortening of telomeres in viable cells cannot be excluded. Primary fibroblasts from individuals with WS have also been observed to exit the cell cycle more frequently than passage-matched control cells (Faragher *et al.*, 1993). Regardless of whether *SGS1* has direct effects on telomere shortening or only on the propensity of cells to arrest as telomeres shorten, our data indicate that *SGS1* has important roles in senescing cells that depend on loss of telomerase because *TLC1 sgs1* mutant cells do not show significant cell cycle arrest.

The localization of WRN with telomere factors, at least in ALT cells, and the functional experiments with telomerase-deficient *sgs1* mutants, together with previous reports of telomere abnormalities in Werner cells (Schulz *et al.*, 1996; Tahara *et al.*, 1997), support a role for telomere abnormalities in the genesis of the WS phenotype. Also supporting this idea are the recent demonstration that WRN interacts with the Ku protein (Cooper *et al.*, 2000; Li and Comai, 2000), which is part of telomeric chromatin in human cells (Hsu *et al.*, 1999), and also the demonstration that WRN, assisted by RPA, can unwind complexes formed of telomeric DNA *in vitro* (Ohsugi *et al.*, 2000). If telomerase masks a role for WRN at telomeres, as it does for *SGS1*, then WS phenotypes would be expected to occur in slowly dividing tissues that lack telomerase, rather than rapidly proliferating tissues that contain progenitor cells with active telomerase. This prediction holds well for much of WS pathology, e.g. in the dermis, vascular endothelium, adipose tissue and bone (Epstein *et al.*, 1966; Shen and Loeb, 2000). It is also consistent with the absence of pathology related to cell loss in the epidermis, bone marrow and gastrointestinal tract that would otherwise be expected if telomerase-positive progenitors could not replenish these tissues.

Some features of WS, like premature hair loss, are not readily explained by this hypothesis, but may be a consequence of other WRN functions or may be secondary to defects in other tissues. Our results also suggest that the relative unimportance of WRN in mice (Lombard *et al.*, 2000) could be explained by the dramatic differences in mouse and human telomere biology (reviewed by Sherr and DePinho, 2000; Wright and Shay, 2000). Mouse chromosomes have telomeres that are about five times longer than human, and mouse tissues have more abundant telomerase activity (Chadeneau *et al.*, 1995; Prowse and Greider, 1995). Moreover, mice are relatively unaffected by the loss of telomerase: cultured mouse fibroblasts and intact mice lacking the mTR telomerase RNA show no evidence of senescence induced by telomere shortening and do not appear to incur pathologies until the point where telomeres become short enough to induce crisis (Blasco *et al.*, 1997; Lee *et al.*, 1998; Rudolph *et al.*, 1999). It will be informative to test whether mice lacking both mTR and WRN develop synthetic defects.

*SGS1* functions in parallel with *RAD51* in the generation of survivors of senescence, placing *SGS1* in the *RAD50*-dependent survivor pathway (Le *et al.*, 1999). *SGS1* is also required for the emergence of type II survivors. Even though *SGS1* can be disrupted in preformed type II survivors, the resulting cells have a very poor growth rate, which could explain the inability of *sgs1 tlc1* mutants to generate type II survivors. These observations are consistent with the recent demonstration that *RAD50* is also required for the emergence of type II survivors, while *RAD51* appears to be required for the emergence of type I survivors (Teng *et al.*, 2000).

The function of *SGS1* in a *RAD51*-independent survivor pathway raises the possibility that *SGS1* might participate in a break-induced-replication (BIR) mechanism at telomeres. In BIR, a chromosome end invades a recipient chromosome and copies the recipient by replication to the end of the chromosome (Haber, 1999). BIR is *RAD51* independent in yeast (Malkova *et al.*, 1996) and has been suggested as a mechanism of telomere maintenance in survivors (Le *et al.*, 1999). After invasion, the BIR intermediate resembles structures believed to reinitiate replication from stalled replication forks (Haber, 1999), and there is evidence that both Sgs1p and WRN localize to and function at stalled replication forks (Constantinou *et al.*, 2000; Frei and Gasser, 2000). The physical interaction of WRN with the replication proteins proliferating cell nuclear antigen, polymerase  $\delta$  and RPA (Brosh *et al.*, 1999; Huang *et al.*, 2000; Kamath-Loeb *et al.*, 2000) is also consistent with this notion. In addition, Sgs1p and WRN are potent G-quadruplex DNA helicases *in vitro* (Fry and Loeb, 1999; Sun *et al.*, 1999). Perhaps during a BIR-like event, the long TG<sub>1-3</sub> repeats of type II survivors are prone to forming G-quadruplexes, or related secondary structures, which require processing by Sgs1p. The poor growth of type II survivors that lack *SGS1* supports this idea.

Might WRN be involved in human ALT? It is intriguing that WS and RTS patients are particularly prone to sarcomas (Lindor *et al.*, 2000), and that sarcomas appear to be more susceptible to ALT pathways than are carcinomas (Colgin and Reddel, 1999), the latter being typically positive for telomerase. Telomere maintenance

by subtelomeric repeat amplification can occur in mouse cells, suggesting that a mammalian equivalent of the type I survivor pathway may exist (Niida *et al.*, 2000). Moreover, we have identified an unusual ALT WS fibroblast line that has very short telomeric repeats but amplified telomere-associated sequences (our unpublished data), also reminiscent of yeast type I survivor telomeres. In yeast, the type I pathway is associated with unstable growth, possibly reflecting genome instability. If loss of WRN or RTS biases ALT cells toward a type I pathway, this might contribute to genome instability and the occurrence of sarcomas in WS and RTS. It will be informative to examine the telomeres of tumor cells from individuals with these disorders.

In summary, our findings indicate that the Sgs1p and WRN helicases play important roles in telomere biology, suggesting that a telomere defect is relevant to the pathophysiology of WS and perhaps other RecQ helicase diseases.

## Materials and methods

### Yeast strains and methods

Yeast were cultured at 30°C with standard media. All strains were isogenic and derived from JKM111 and the *rad51* and *rad50* derivatives JKM114 and JKM116 (Le *et al.*, 1999). *SGS1* was deleted using the *sgs1 $\Delta$ ::hisG-URA3* construct pPP69 (Park *et al.*, 1999). *TLC1* was deleted using PCR-mediated replacement of transcript bases 141–1212 with a kanMX G418-selectable marker (Guldener *et al.*, 1996). Double and triple mutants were constructed by deleting one copy of *TLC1* in diploids heterozygous for *sgs1*, or *sgs1* and *rad50* or *rad51* followed by sporulation. All genotypes were confirmed by auxotrophy/G418 resistance and by PCR amplification of fragments that crossed mutation breakpoints and were thus different sizes for wt and mutant alleles. Senescence experiments were performed starting with spore products from reference plates (estimated population doubling of 30). Liquid senescence experiments were as described (Le *et al.*, 1999), with rounds of inoculation of  $2 \times 10^6$  cells into 5 ml of YPD followed by growth for 23 h, counting of cells with a Coulter counter, and re-inoculation. Plate senescence experiments were performed using a mixture of five colonies ( $10\text{--}20 \times 10^6$  cells) and growth for 3 days per restreak. Genotypes of survivors were confirmed as for starting spore products. Continued passage of survivors in liquid was performed by diluting saturated cultures 1000-fold serially every 1.5 (*tlc1*) or 3 days (*sgs1 tlc1*), each passage counted as 10 doublings. Viability of cells senescing in liquid culture was assessed by plating 1000 cells, from each of the six cultures analyzed in Figure 2A, and observing cells/colonies using microscopy after 36 h of growth. At least 100 cells/colonies were counted per culture, and isolated large single- and double-budded cells were considered arrested.

### Human cells

WI38 75.1, a clone of WI38 fetal lung fibroblasts immortalized with the SV40 large T antigen, were obtained from the Coriell cell repository, and were grown as described (Marciniak *et al.*, 1998). The ALT phenotype of WI38 75.1 was determined using standard telomeric TRF blots and TRAP assays for telomerase (Counter *et al.*, 1998). HeLa S3, WI38, VA13, AG07066, U2OS, Saos2 and BJ cells were obtained from the American Type Culture Collection or the Coriell cell repositories. LM216J and hTERT-immortalized BJ fibroblasts were as described (Murnane, 1986; Hahn *et al.*, 1999).

### Telomere analysis

Southern analysis was performed essentially as described (Le *et al.*, 1999). Briefly, digested yeast genomic DNA was separated on 1% agarose gels, transferred to Hybond-XL (Amersham) and hybridized to random-primed poly(dG)–(dT) or a 784 bp PCR-generated Y' fragment (GACTGCAGGG...GAAAGCAGGG) lying between the terminal *XhoI* site to the start of the terminal TG<sub>1-3</sub> repeats. Washed blots were visualized using a Molecular Dynamics PhosphorImager. To measure initial telomere shortening rates, entire spore colonies were dissected

from plates, resuspended in 5 ml of liquid YPD and grown to an OD of 1–3. Cell number was determined with a Coulter counter and population doublings from the original spore calculated as  $\log_2(\text{final cell number})$ . Data files generated from telomere blots probed with the 784 bp Y' fragment (above) were analyzed for mean TRF length using NIH Image and Microsoft Excel software. The weighted average was used to determine mean telomere length:  $\Sigma(\text{OD}_i \times \text{length}_i) / \Sigma(\text{OD}_i)$ . *P* values for differences between telomere lengths and rates of shortening were calculated using a two-tailed, unpaired two-sample *t*-test.

### Flow cytometry

Log-phase cells were prepared as described (Mills *et al.*, 1999), except that they were sonicated gently prior to fixation, all centrifugations were at 2000 *g* and digestions were at 50°C. Propidium iodide-stained cells were analyzed on a Beckman-Dickson FACScan using CellQuest and ModFit software; 30 000 cells were acquired per sample.

### Immunofluorescence

For yeast, log-phase cells were fixed in formaldehyde and spheroplasted with zymolyase as described (Mills *et al.*, 1999). DNA was stained with DAPI and microtubules were visualized using the mouse anti-TUB2 antibody BIB2 and an anti-mouse Cy3 conjugate (Amersham) diluted 1:200 in phosphate-buffered saline/1% bovine serum albumin/0.1% Triton X-100. Digital images were obtained using a SPOT RT CCD camera and software. Human fibroblasts were grown on multi-welled slides, fixed and stained for WRN using an affinity-purified rabbit anti-WRN antibody as described (Marciniak *et al.*, 1998). Mouse monoclonal anti-TRF-2 antibodies (Imgenex) and anti-PML antibodies (Santa Cruz Biotechnology) were used at dilutions of 1:800 and 1:200, respectively. Secondary antibodies were a goat anti-rabbit fluorescein isothiocyanate (Vector Labs) at 1:100 and goat anti-mouse Cy3 conjugate (Amersham) at 1:300. Microscopy was performed at the WM Keck Foundation Biological Imaging Facility at the Whitehead Institute on a Zeiss laser scanning confocal microscope, sampling 0.8  $\mu\text{m}$  optical sections.

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