

# A two-step model for senescence triggered by a single critically short telomere

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**Telomeres protect chromosome ends from fusion and degradation<sup>1</sup>. In the absence of a specific telomere elongation mechanism, their DNA shortens progressively with every round of replication, leading to replicative senescence<sup>2</sup>. Here, we show that telomerase-deficient cells bearing a single, very short telomere senesce earlier, demonstrating that the length of the shortest telomere is a major determinant of the onset of senescence. We further show that Mec1p-ATR specifically recognizes the single, very short telomere causing the accelerated senescence. Strikingly, before entering senescence, cells divide for several generations despite complete erosion of their shortened telomeres. This pre-senescence growth requires *RAD52* (radiation sensitive) and *MMS1* (methyl methane sulfonate sensitive), and there is no evidence for major inter-telomeric recombination. We propose that, in the absence of telomerase, a very short telomere is first maintained in a pre-signalling state by a *RAD52-MMS1*-dependent pathway and then switches to a signalling state leading to senescence through a Mec1p-dependent checkpoint.**

In mouse, the presence of a minimal set of very short telomeres is sufficient to trigger replicative senescence<sup>3</sup>. Nevertheless, it is still unclear whether one single, very short telomere is sufficient and, if so, what the critical telomere length is for signalling senescence. To address this question, we created a telomerase-negative budding-yeast strain in which a single telomere was engineered that could have its length abruptly and dramatically reduced without affecting the integrity of its end (Fig. 1). Specifically, we constructed a diploid strain lacking one copy of the telomerase RNA gene *TLC1* and containing a truncated pair of VII-L telomeres, devoid of any natural subtelomeric sequences, to focus on the role of the minimal functional unit of the telomere — the telomeric repeats. The modified VII-L telomere, used previously to monitor various aspects of telomere function<sup>4,5</sup>, contained internal telomeric repeats flanked by recognition

sites for a recombinase. After sporulation, inducing the recombinase during germination resulted in excision without affecting the integrity of the chromosome end. Telomerase-negative colonies were selected by means of the marker used to delete *TLC1*, which is only expressed in haploid cells<sup>6</sup>. Three sets of strains were created containing increasing numbers of internal telomeric repeats: a shortening of approximately 100–120 base pairs (bp) was achieved with the strains named 1 block and 2 block, compared with the control strain, 0 block (Fig. 1c).

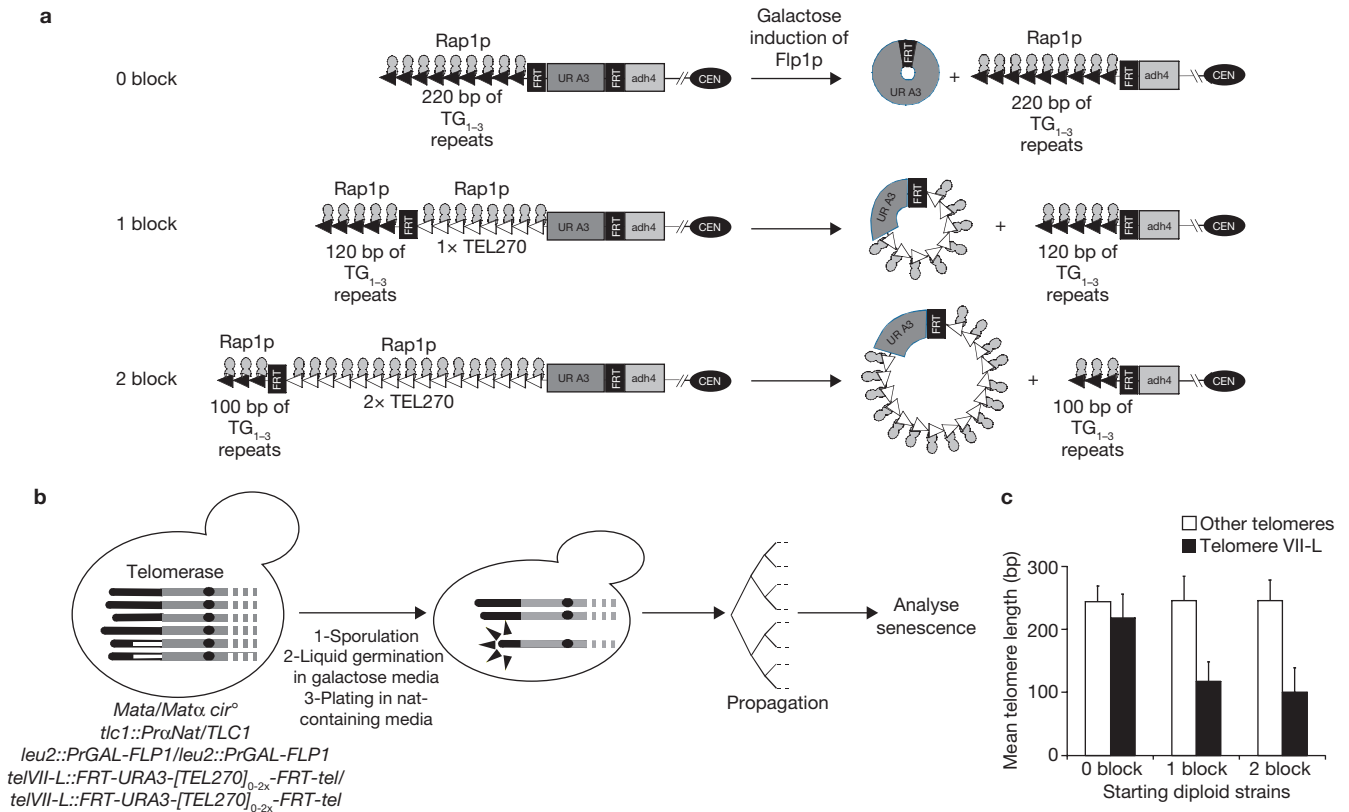
When several independent control spores (0 block) were serially spotted to follow senescence, we observed that, as expected, cell growth was severely impaired at the third passage (Fig. 2a). However, cells containing a single short telomere (1 or 2 block strains), lost the capacity to form colonies at the second passage. Glucose-grown colonies that did not contain a particular shortened telomere showed no difference in the onset of senescence (data not shown). Median viability curves based on cell number and colony size revealed that the shorter the VII-L telomere, the sooner cells lost viability (Fig. 2b; see Methods; Supplementary Information, Table S1). Morphologic inspection and DNA staining showed that a large fraction of cells containing a very short telomere were arrested in G2/M cell-cycle phase (Fig. 2c), indicating that the growth arrest triggered by the very short telomere was similar to true senescence. Thus, the number of generations until a telomerase-negative cell senesces is reduced by shortening a single telomere.

To monitor the timing of the onset of senescence more precisely, we grew cells continuously in liquid culture and measured the number of population doublings undergone by each strain every 24 h (Fig. 2d). As with true senescence, the culture growth rate initially decreased, but then increased with the appearance of rapidly growing survivors<sup>7</sup>. We found that shortening a single telomere accelerated the onset of senescence by approximately 15 population doublings (from 60 to 45 generations, Fig. 2e). This senescence acceleration in strains bearing a short telomere was surprising given that these strains initially have ~100 bp of TG<sub>1-3</sub> repeats on the VII-L telomeres (Fig. 1), which should shorten by 3–4 bp per generation<sup>4</sup>; suggesting that all repeats would be absent in 30 generations. Most of the spores that lost

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**Figure 1** Experimental system. (a) Flp1-dependent excisable blocks of internal TG<sub>1-3</sub> repeats were introduced in the proximity of a new telomere formed at the *ADH4* locus of VII-L<sup>4,31</sup>. Three sets of strains were constructed: 0 block, which served as control; 1 block, which contained 270 base pairs (bp) of internal TG<sub>1-3</sub> and 2 block, which contained two blocks of 270 bp of TG<sub>1-3</sub> repeats. (b) To obtain the shortening in a telomerase-negative context, we created a set of diploid strains homozygous for the modified telomere VII-L (0, 1 or 2 block) and heterozygous for the telomerase RNA (*TLC1/tlc1Δ*). The telomerase RNA gene was deleted using a cassette expressing the nourseothricine resistance marker under the control of an alpha-specific promoter<sup>6</sup>. Nourseothricin-resistant

colonies unable to grow in medium lacking uracil were deleted of telomerase and had a circle of telomeric TG<sub>1-3</sub> repeats excised; they had a telomere tract about half the size of the average telomere. (c) Telomere length in starting diploid strains (yT136, yT137 and yT138, 0 block, 1 block and 2 block, respectively; see Supplementary Information) were estimated by telomere-PCR using an oligonucleotide targeting the telomere-proximal-FRT region of telomere VII-L (oT155) and an oligonucleotide targeting a subtelomeric element, the long Y' element<sup>10</sup>, which is conserved in many sub-telomeres of *S. cerevisiae* (oT100). See Methods for details. Error bars represent s.d., *n* = 3 for all samples of other telomeres and *n* > 4 for telomere VII-L samples.

2 blocks of repeats grew for 45 generations, indicating that the truncated VII-L telomere is somehow maintained in a functional state in pre-senescent cells or that a second event is required to trigger senescence.

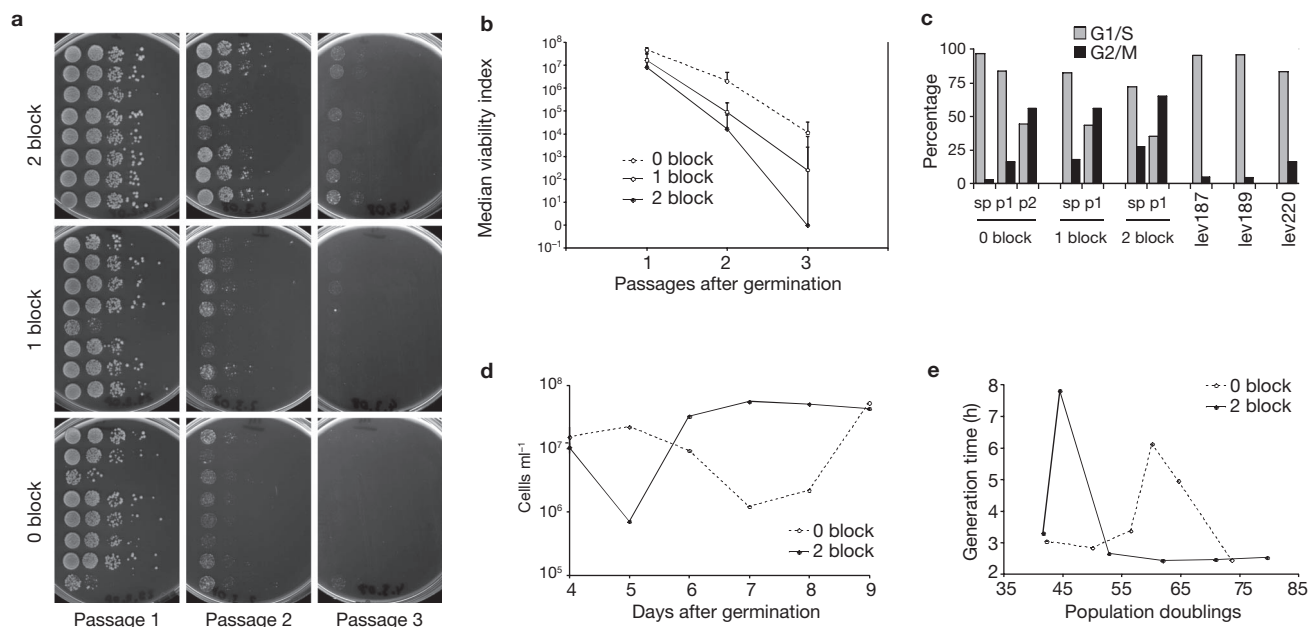
To test whether a second event contributes to senescence, we followed the growth of a given spore in eight independent senescence assay replicates and found that the viability profiles were almost identical between replicates from control spores (Supplementary Information, Fig. S1a), which is similar to previous findings<sup>8</sup>. Thus, the timing of the second event is apparently fixed, raising the possibility that replicative senescence is triggered by a structural switch of a single very short telomere from a pre-signalling state to a senescence-signalling state.

To determine whether the structure of the very short telomere switches to a senescence-signalling state, the length of telomere VII-L was measured by telomere-PCR<sup>9</sup>. In addition to the modified VII-L telomere, most telomeres in these strains bear a sub-telomeric repeat called Y', which was used to measure their length<sup>10</sup>. We observed that Y' and modified-VII-L telomeres of 0 block strains progressively shortened (Fig. 3a). As expected, the length of the modified-VII-L telomere was shorter than the other telomeres in the 1 and 2 block strains. Strikingly, the length of the very short telomere in 1 or 2 block strains was approximately constant in the original colony, in the dividing cells in passage 1 and in the largely senescent population in

passages 2 and 3. Thus, the switch to a senescence-signalling state does not seem to involve a further reduction in the size of the very short telomere. To determine the structures of the pre-signalling and senescence-signalling telomeres, modified-VII-L telomeres were cloned and sequenced (Fig. 3b). Surprisingly, many DNA ends did not contain any telomeric repeats even though these telomeres were cloned from colonies just after germination, when most cells are viable (Fig. 3a, spore colony).

To investigate how cells sustain their proliferation despite the complete or nearly complete loss of telomere sequence, we investigated whether pre-senescent cells adapted to non-reparable double-strand breaks<sup>11</sup>. When Ptc2, a critical phosphatase involved in this process<sup>12</sup>, was deleted, we saw no reduction in cell viability (Supplementary Information, Fig. 1b–c and Table S1). Thus, adaptation is unlikely to have a key role in the viability of cells bearing the very short telomere.

To test whether the senescence-pre-signalling telomere is maintained by homologous recombination, we examined the role of *RAD52*. In both the 0 and 2 block conditions, the viability of *rad52Δ* spores was severely compromised compared with wild-type colonies (Fig. 3c; Supplementary Information, Fig. 1d and Table S1), consistent with previous work<sup>7</sup>. Moreover, the loss of viability correlated with an increased accumulation of G2/M cells (Supplementary Information, Fig. 1e). Thus, the absence of



**Figure 2** Senescence is advanced in the presence of a critically short telomere. (a) Senescence assay on spores containing a critically short telomere (1 and 2 block) and a control (0 block). Spore-colonies from diploids obtained as described in Fig. 1b (see Supplementary information) were resuspended to equal concentrations and serial 10-fold dilutions were spotted on rich-media plates. After two days at 30 °C the process was repeated starting with a mixture of cells from the most concentrated spot. (b) Median viability index measured from samples in panel a taking into account both the capacity to form colonies and the size of the colonies (see Methods). Error bars represent s.d.,  $n = 8$ . See Supplementary Information, Table S1 for further statistical analysis of this data. (c) Quantification of the cell-cycle distribution of DAPI-stained cells from clones in panel a. Exponentially

growing cultures of indicated genotypes were obtained from the spore colony (sp), first passage (p1) or second passage (p2) and stained with DAPI as described in Methods. Telomerase-positive strains<sup>4</sup> were used as controls (see Methods). Data from two independent spores were cumulated. (d) Spore colonies were grown overnight in a small volume of YPD, diluted to  $1 \times 10^5$  cells ml<sup>-1</sup> per 100 ml culture and grown for 22 h. Each day, the cell culture was diluted to  $1 \times 10^5$  cells ml<sup>-1</sup>. The mean cell concentration was recorded each day, using a hemacytometer, from two independent spores of the indicated genotypes. (e) Data from Fig. 1c was reformatted to plot generation time (time taken for the population to double) as a function of the number of population doublings, with the assumption that by the third day all spores had undergone 35 generations.

*RAD52* and telomerase activity severely reduces the number of divisions cells can undergo before entering into senescence. Both *RAD52* and *rad52Δ* cells had similar telomere lengths (Fig. 3a, right panels), despite the fact that *rad52Δ* cells had undergone fewer population doublings. Thus, the role of Rad52 in pre-senescence does not seem to involve a major modification of the length of the very short telomere.

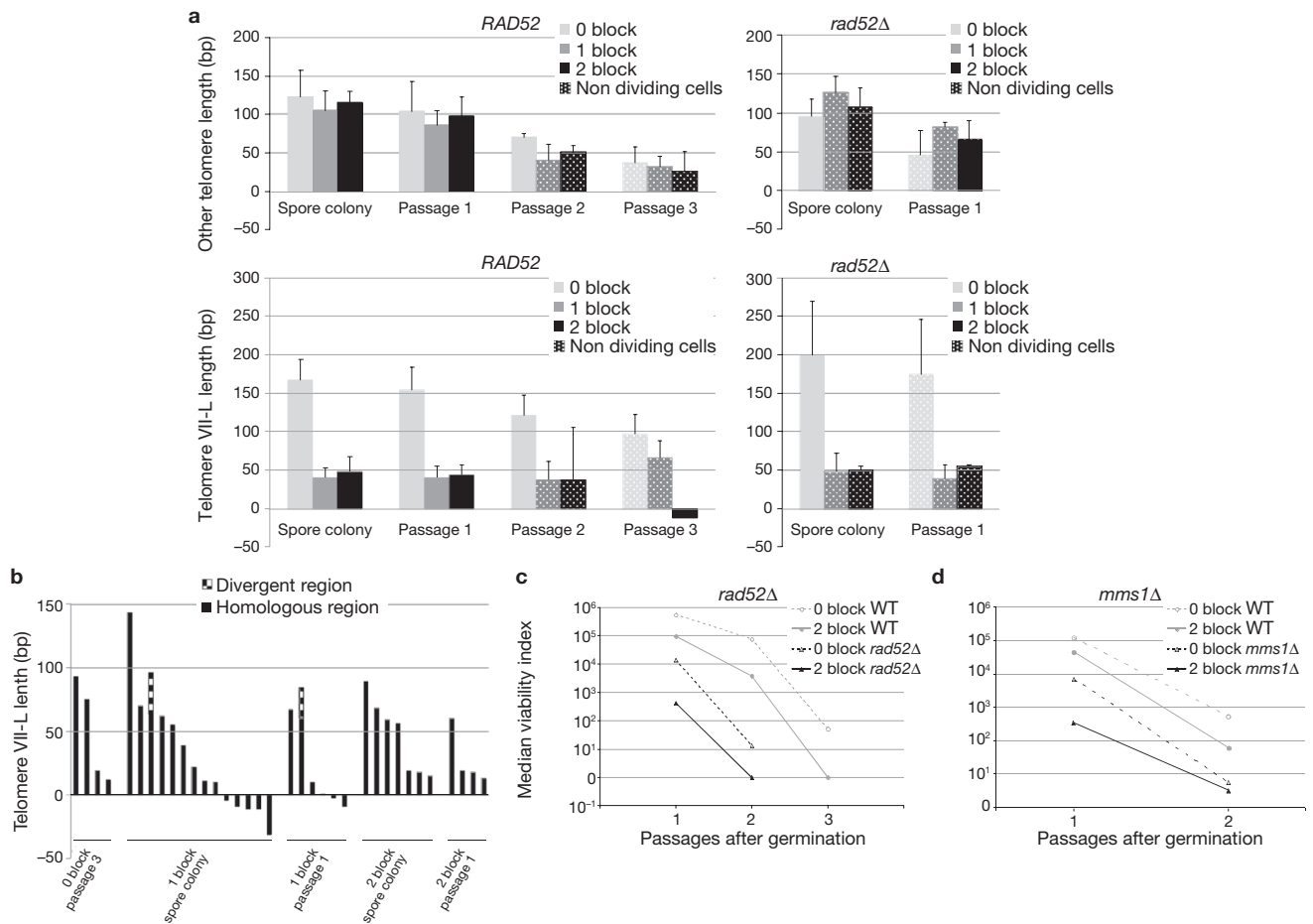
To determine whether *RAD52* supports cell growth by promoting recombination between the very short telomere and the other telomeres, the sequences of the modified-VII-L telomeres from *RAD52 tlc1* strains were examined for the addition of new sequences. Only 2 of the 35 very-short cloned telomeres from pre-senescent cells had acquired divergent sequences (Fig. 3b), which is similar to the frequency observed previously for median length telomeres<sup>13</sup>. Thus, the maintenance of the very short telomere in a pre-senescent state cannot be explained simply by an increased rate of inter-telomeric or unequal intra-telomeric exchanges. These data also show that the recombination-based mode of telomere maintenance occurring in post-senescent survivors<sup>7,14</sup> is not operational in pre-senescent cells.

Rad52 functions in several pathways of homologous recombination including the repair of double-strand breaks and the repair of stalled replication forks<sup>15</sup>. To determine if one of these pathways is involved in the maintenance of the very short telomere, we tested the effect of mutating *MMS1*, a gene involved in the repair of stalled forks but not of double-strand breaks<sup>16-19</sup>. We found that *mms1Δ* had an effect similar to that of *rad52Δ* (Fig. 3d; Supplementary Information, Fig. 1f and Table S1). Therefore, a function common to the protein products of *MMS1* and *RAD52* seems to

be required to maintain viability of pre-senescent cells. Because both genes are required to repair stalled replication forks, we speculate that very short telomeres are particularly difficult to replicate and require homologous recombination for its completion.

Next, we asked whether the PI(3)K-related protein kinase Mec1-ATR is required for accelerated senescence in the presence of a very short telomere. To compensate for the lack of essential *MEC1* function, *SML1* was also deleted in the starting diploid strains<sup>20</sup>. Compared with *MEC1 SML1* strains, we observed that the loss of *mec1Δ sml1Δ* strain viability was delayed by approximately one passage in the absence of G2/M cell-cycle arrest (Fig. 4a; Supplementary Information, Fig. 1g-h and Table S1), confirming previous observations of the involvement of Mec1 in the signalling of senescence<sup>8,21</sup>.

No statistically significant differences in times of senescence were observed between the *MEC1 SML1* and *mec1Δ sml1Δ* 2 block strains (strains with very short telomeres; Fig. 3a; Supplementary Information, Fig. 1g), in the first two passages. However, most *mec1Δ sml1Δ* 2 block strain clones were still viable after the third passage and their viability curves resemble the *mec1Δ sml1Δ* control strains with a 0 block telomere. This ability of cells to form colonies was not due to post-senescent survival as after the fourth passage most cells stopped growing in the absence of a marked G2/M arrest (Supplementary Information, Fig. 1h). Therefore, lack of Mec1 and Sml1 results in the bypassing of accelerated senescence due to the presence of a very short telomere. We conclude that a senescence-signalling telomere acquires the ability to activate Mec1.



**Figure 3** *RAD52*- and *MMS1*-dependent growth of pre-senescent cells can be uncoupled from inter-telomeric recombination. **(a)** Genomic DNA was prepared from overnight cultures of successive passages of sister spores from wild type (*RAD52*) or *rad52* $\Delta$  strains, after excising 1 or 2 blocks of telomeric TG<sub>1-3</sub> repeats or no telomeric sequences (0 block). Telomere-PCR was performed using oligonucleotide  $\alpha$ T157 that targets *ADH4* sequences (VII-L telomere) or  $\alpha$ T100 that targets Y' telomeres (other telomeres). The estimated mean length of pure TG<sub>1-3</sub> repeats were measured in at least five samples. Error bars indicate s.d. Other telomeres,  $n = 4$ , except 1 block spore colony where  $n = 3$ . VII-L telomeres,  $n = 4$ , except 1 passage 3 strain where  $n = 3$ . **(b)** Telomere

PCR of VII-L telomeres from the representative samples indicated were cloned and sequenced. The length of pure TG<sub>1-3</sub> repeats is depicted. Negative values correspond to telomeres that were truncated in the subtelomeric region. TG<sub>1-3</sub> sequences were aligned as described previously<sup>13</sup> and divergent repeats were detected. **(c-d)** Diploids described in Fig. 1b containing one allele of the indicated mutations were sporulated and germinated in the presence of galactose to induce telomere shortening (WT, wild type). Median viability was measured as described in Fig. 2 using the spots shown in Supplementary Information, Fig. 1 d and f. See Supplementary Information, Table S1 for further statistical analysis.

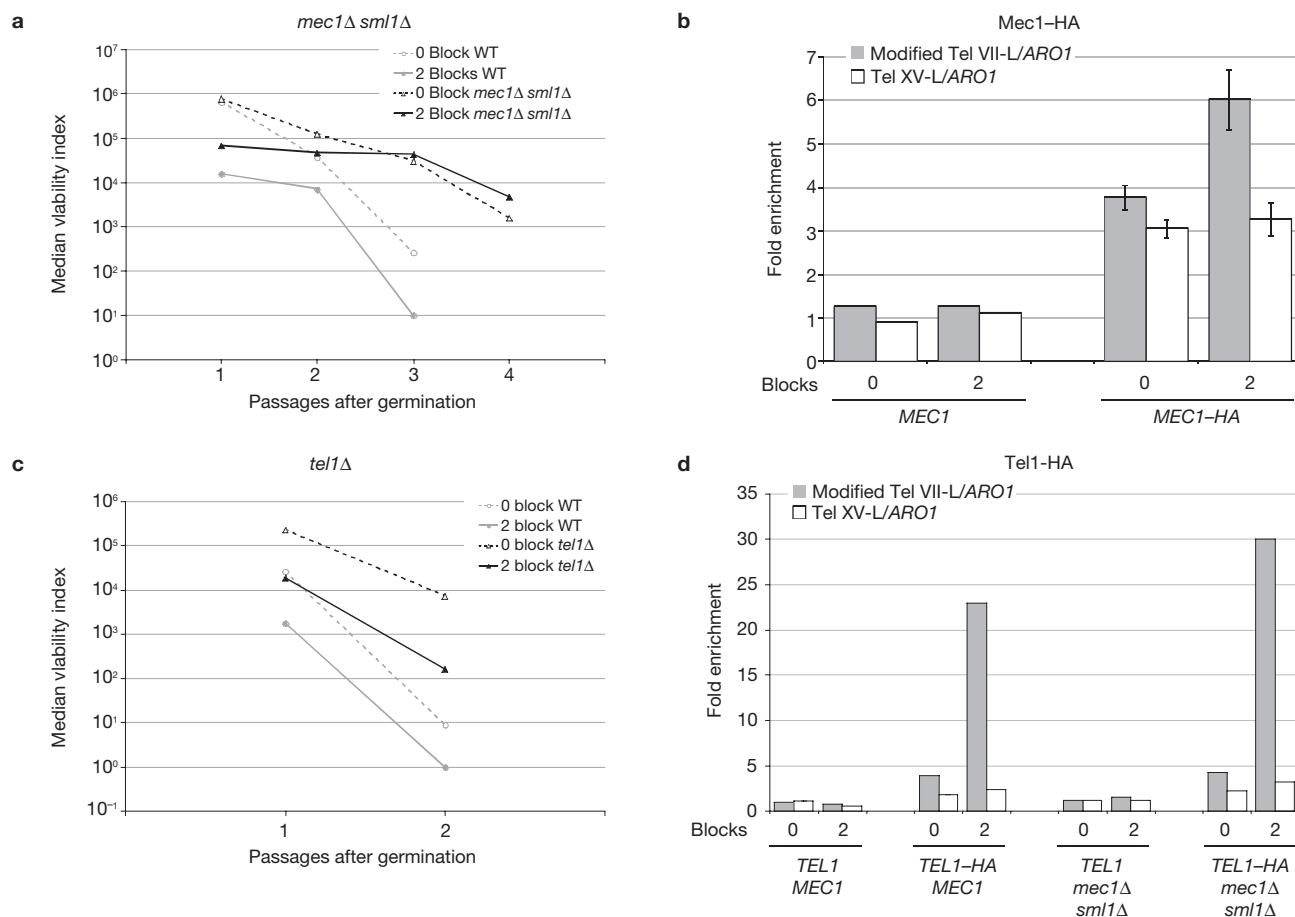
To test whether Mec1 senses the senescence-signalling telomere through direct binding, we generated telomerase-negative spores carrying haemagglutinin-tagged versions of Mec1 (Mec1-HA) and a single shortened telomere. Chromatin immunoprecipitation (ChIP) showed that Mec1 was enriched at telomeres in pre-senescent cells, but enrichment at the very short telomere was approximately twice as efficient (Fig. 4b). This supports the idea that Mec1 interacts physically with the signalling telomere, thereby sensing and transducing a specific signal emanating from it to cause G2/M cell-cycle arrest.

Tel1 (telomere maintenance) binds to short telomeres<sup>5,22-24</sup> but its impact on senescence is still elusive<sup>8,21,25</sup>. To gain an insight into the role of Tel1 in senescence, we introduced the *tel1* $\Delta$  mutation in our system. We noticed that *TEL1* 0 block cells descending from a *TEL1*/*tel1* $\Delta$  heterozygote were senescing earlier than those descending from *TEL1*/*TEL1* diploids, allowing only two passages to be analysed. Similarly to previous observations<sup>21,25</sup>, *tel1* $\Delta$  spores senesced later than wild-type (*TEL1*) sister spores even in the presence of a very short telomere (Fig. 4c; Supplementary Information,

Fig. 1i and Table S1), in contrast to *mec1* $\Delta$  *smi1* $\Delta$  cells (Fig. 4a). Thus, *TEL1* promotes replicative senescence but its loss does not abolish the accelerated senescence caused by a very short telomere.

We also generated telomerase-negative spores carrying tagged versions of Tel1 (Tel1-HA) and a single shortened telomere. ChIP showed that, consistent with previous results<sup>5,23,24</sup>, Tel1 efficiently binds a very short telomere, in the presence or absence of Mec1 (Fig. 4d). Tel1 enrichment at the short telomere was much more pronounced than Mec1. This may result from a difference in immunoprecipitability between the two proteins. Alternatively this could reflect the possibility that Tel1 binds to the short telomere in pre-senescent dividing cells, whereas Mec1 binds only in senescent non-dividing cells.

The strong association of Tel1-HA with the shortened telomere before the first passage also indicates that Tel1 association does not result in senescence. These data together with recent work showing that a single double-strand break does not induce a Tel1-mediated checkpoint<sup>26</sup>, identify Tel1 as an unlikely candidate for the checkpoint protein that induces senescence.



**Figure 4** Tel1p promotes senescence and Mec1p transduces a signal from the very short telomere. (a, c) Diploids described in Fig. 1b, containing one allele of the indicated mutations were sporulated and germinated in the presence of galactose to induce telomere shortening. Median viability was measured as described in Fig. 2 using the spots shown in Supplementary Information, Fig. 1g and i. See Supplementary Information, Table S1 for statistical analysis. WT, wild type. (b, d) Spore colonies of the indicated genotype, obtained as described in Fig. 1b, with

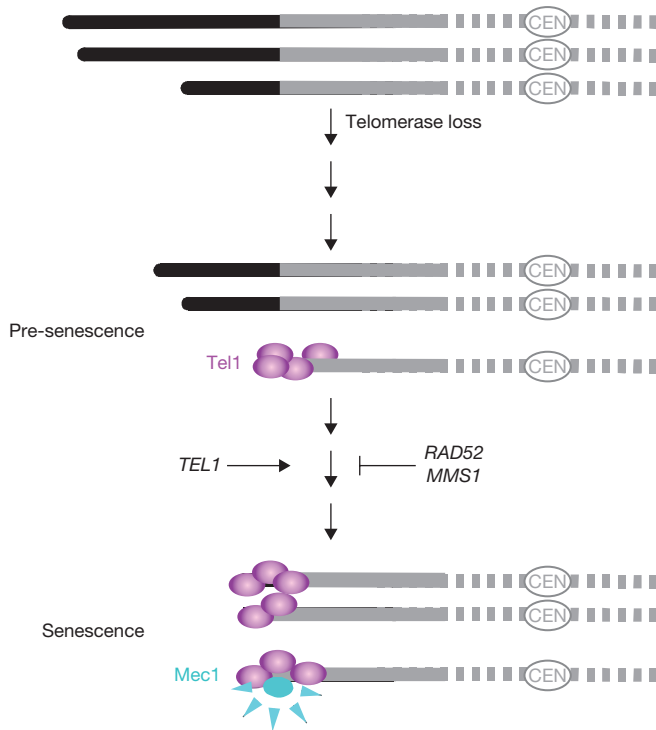
Our results show that a single, very short telomere determines the onset of replicative senescence. Moreover, dissection of the events occurring at this single, short telomere before and during senescence reveals a two-step mechanism (Fig. 5). First, the very short telomere is maintained in a pre-signalling state through a recombination pathway involving Rad52 and Mms1 that preserves the sequence of individual telomeres. Then, it switches to a senescence-signalling state where it induces cell-cycle arrest by binding to Mec1.

Intriguingly, we were unable to detect any significant differences in telomere length that could account for the switch from the pre-signalling to the senescence-signalling state. Moreover, some pre-signalling telomeres might have completely lost their telomeric repeats, suggesting that capping functions can be epigenetically maintained for few generations after a complete telomeric DNA erosion<sup>27</sup>. In any case, we show that this pre-senescent state is maintained due to a *RAD52*- and *MMS1*-driven activity that does not result in inter-telomeric recombination or intra-telomeric unequal recombination. In further agreement with a specific role of Rad52 in telomere maintenance, a related paper by Khadaroo *et al.* (published in this issue; *Nature Cell Biol.*, doi: 10.1038/ncb1910)<sup>28</sup> shows that Rad52 is rapidly recruited to a very short telomere in telomerase-negative cells. Overall, we propose that a very short

2 blocks or no blocks were inoculated in rich medium and grown exponentially overnight. Chromatin was immunoprecipitated with anti-HA antibodies and analysed by quantitative PCR using primers targeting the telomere VII-L, the telomere XV-L and the internal locus *ARO1*. Enrichment values are presented as the ratio of a given telomere enrichment (immunoprecipitated / input) to the enrichment of the *ARO1* locus (immunoprecipitated / input). Error bars indicate s.d. Values obtained from three independent spores.

or even an eroded telomere can be maintained for some generations in a pre-senescence-signalling state through sister chromatid recombination. As this pathway is usually involved in the repair of arrested replication forks, we suggest that a very short telomere could be sensitive to replication fork progression. Both the specificity of the *MEC1* deletion phenotype in cells containing a very short telomere and recognition of the very short telomere by Mec1-Ddc2 and replication protein A (as shown in this study and in related work by Khadaroo *et al.*)<sup>28</sup> suggest that the primary signal triggering senescence could be a long stretch of single-stranded DNA.

The relative contribution of ATR-Mec1 and ATM-Tel1 to telomere dysfunction-induced checkpoints remains to be defined in mammalian models. The finding that checkpoint activation in intestinal stem cells harbouring dysfunctional telomeres involves Exo1-mediated activation of ATR-Mec1 (ref. 29) and that fibroblasts in the dermis of baboons rarely contain more than one telomere-damage focus, even in very old animals<sup>30</sup>, suggest that, as shown here in yeast, mammalian cells have the ability to activate an ATR-Mec1 checkpoint through a signal arising from a single critically short telomere. We propose that this 'single-telomere checkpoint' reflects a conserved property of eukaryotic cells that contributes to genome stability and cellular homeostasis.



**Figure 5** A two-step model for senescence triggered by a single, critically short telomere. In the absence of telomerase, cells containing a critically short telomere have prolonged viability. This allows the definition of a pre-senescent state that can be modulated by genetic manipulations: *RAD52* and *MMS1* deletions abrogate this state and *TEL1* deletion prolongs it. In the presence of telomerase, the telomere-bound Tel1 would lead to telomerase recruitment and telomere elongation<sup>5,22–24</sup>. In its absence, telomeres become a substrate for Rad52- and Mms1-dependent processes that protect the telomeres and may abrogate the signalling pathway that activates senescence (for example, sister chromatid recombination). We demonstrate that Mec1 binds, senses and transduces a signal from the very short telomere. We speculate that pre-senescent cells switch to senescent cells when single-stranded subtelomeric sequences are sufficiently exposed to activate the Mec1 pathway. This is expected to occur initially at the shortest telomere.

*Note added in proof: a related manuscript by Khadaroo et al. (Nature Cell Biol., doi: 10.1038/ncb1910) is published in this issue.*

## METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturecellbiology/>.

*Note: Supplementary Information is available on the Nature Cell Biology website.*

## ACKNOWLEDGEMENTS

We thank R. Wellinger and L. Rudolph for fruitful suggestions, G. Yvert and C. Lopes for advice in statistical analysis and A. Jacquier for sharing unpublished material. We also thank T. Petes, M. P. Longhese and S. Marcand for strains and plasmids. P.A. gratefully acknowledges the financial support of the Lebanese National council for Scientific Research (CNRSL) and the Association pour la Recherche sur le Cancer (ARC). This work was supported by La Ligue Contre le Cancer (E.G. and V.G., équipes labellisées), the INCa programmes TELINCA and TELOFUN (E.G. and V.G.), The Danish Agency for Science, Technology and Innovation (M.L.), the Villum Kann Rasmussen Foundation (M.L.) and the National Institutes of Health (K.R.).

## AUTHOR CONTRIBUTIONS

P.A., P.L. and M.T.T. carried out and analysed the experiments. E.G. and M.T.T. designed and directed the project with the contribution of K.W.R., M.L. and V.G.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## METHODS

**Strains.** All strains had a W303 background (see Supplementary Information for a list of strains). They were generated from *lev187*, *lev189*, *lev220* (MATa *ura3-1 trp1-1 leu2-3, 112::Gal-FLP1-LEU2 his3-11, 15 can1-100 ade2-1 telVIIadh4::FRT-URA3-[tel270]<sub>0-2x</sub>-FRT-tel*)<sup>4</sup>, a wild-type W303 strain bearing the *tlc1Δ::PαNatMX4* — constructed as described previously<sup>6</sup> and strains from the laboratories of T. Petes<sup>32</sup> (Duke University Medical Centre, NC, USA) and M-P. Longhese<sup>33</sup> (University of Milano-Bicocca, Milan, Italy). Details are available on request.

**Generation of a short telomere.** To obtain a short telomere in a telomerase-negative context, diploids were sporulated in 2% potassium acetate plates for 3 days at 30 °C until sporulation efficiency reached more than 20%. The mixture was then resuspended in water (100 μl) and equal amounts of cells were used to inoculate 2 ml of YPGal–Raff (2% galactose and 1% raffinose) or YPD liquid media at approximately 1–5 × 10<sup>6</sup> tetrads ml<sup>-1</sup>. After 6 h of germination at 30 °C, several different dilutions of the germination mixture were plated in YPD supplemented with nourseothricin. Colonies were allowed to form for 40 h at 30 °C. Only 1–10% of cells germinated in galactose-containing media formed colonies, although for cells germinated in YPD the colony formation capacity is 80–100%. As the strains containing no additional telomeric repeats (0 block) also showed 1–10% germination, the process of telomere excision did not appear to alter germination. Colonies on plates of similar colony density (typically 500–1000 colonies per plate) were then tested for uracil prototrophy, or any other marker, by streaking a very small portion of the colony on appropriate media. Identification of spores containing HA tags or the *smi1Δ* and *tel1Δ* alleles was performed by PCR (oligonucleotide sequences are available on request).

**Semi-quantitative senescence assay.** Nourseothricin-resistant, ura-negative colonies were resuspended in water in microtitre plates and the OD (optical density) at 600 nm was measured. Cell concentrations were determined using a standard curve and dilutions were performed to obtain suspensions of equal cell concentrations. In most experiments, this initial concentration was 400,000 cells ml<sup>-1</sup>. When the spore colonies were very small (for example, *rad52Δ* colonies), a lower initial concentration was used. In all cases, the concentration of the wild-type controls and mutant strains within an experiment were equal, and cells were plated at the same time. Spots of 10 × serial dilutions of these suspensions were grown for 2 days at 30 °C in YPD supplemented with nourseothricin. The most concentrated spots (representing a mixture of many colonies) were then resuspended, diluted to a known concentration and re-spotted onto plates, and the procedure was repeated for the next serial passage.

Telomerase-negative cells with short telomeres show two phenotypes: reduced plating efficiency and slow colony growth. To quantify both phenotypes simultaneously, we calculated a viability index for each spore colony, which was based on both the number of colonies in the spot with the highest dilution and the area covered by each isolated colony (which was reduced for slow-growing cells). Thus, the viability index is the area (mm<sup>2</sup>) of the colonies from the spot containing isolated colonies (measured using ImageJ)<sup>34</sup> multiplied by the dilution factor of the spot and normalized to the initial concentration of cells. Passage 1 viability index corresponds to the growth potential of cells contained in the spore-colony; passage 2 viability index corresponds to cells of colonies from passage 1, etc. Within the same experiment and passage, differences in the set of viabilities indexes between different genotypes

were statistically tested with the Wilcoxon rank sum test (Mann-Whitney) using package R v2.6.1 (ref. 35) or XLstat (v2008.1.01).

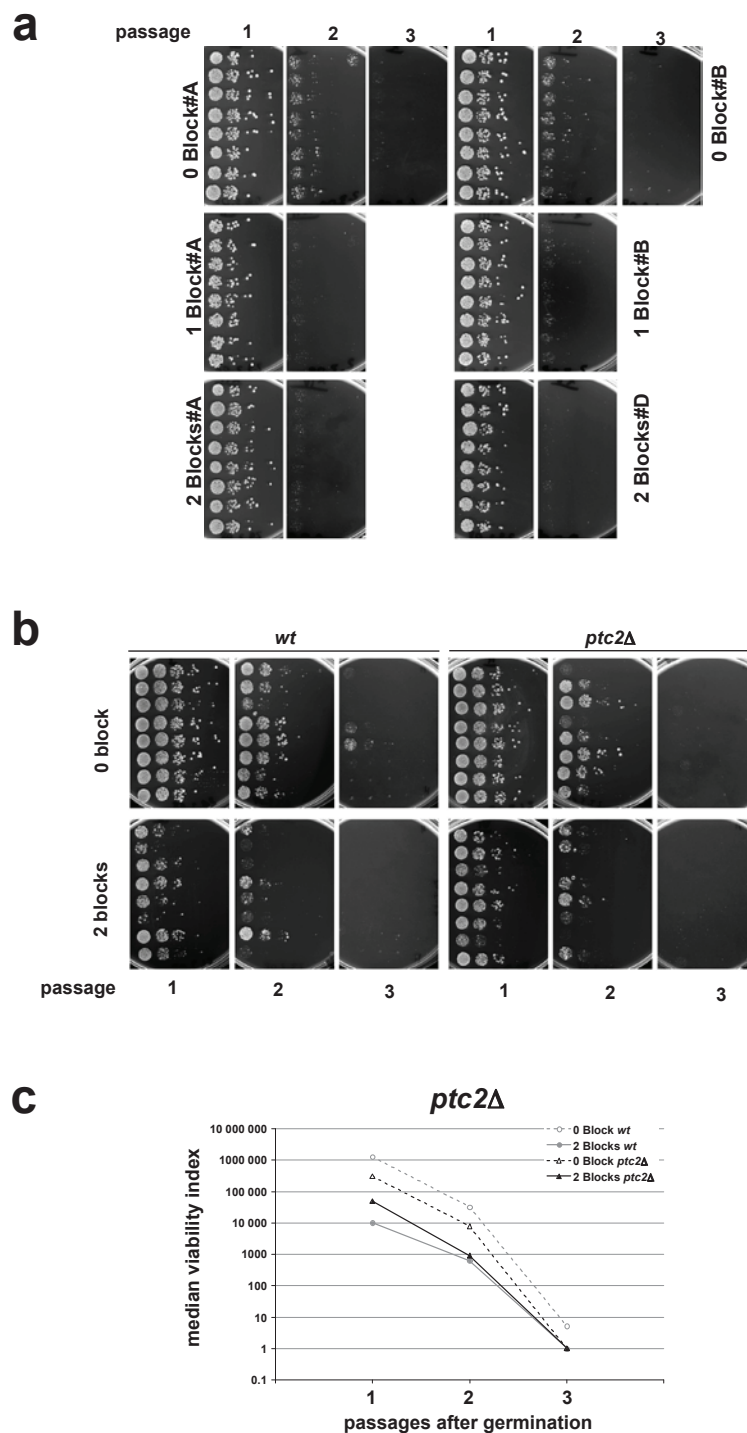
**Microscope analysis.** Exponentially growing cells were fixed with 3.7% formaldehyde for 45 min, treated with zymolyase (2.5 mg ml<sup>-1</sup>) for 20 min at 37 °C and applied to slides coated with 0.1% polylysine. The slides were washed with 1 × PBS and then incubated in DAPI (0.5 mg ml<sup>-1</sup>) for 1 h. After briefly washing and fixing with 100% ethanol, slides were mounted using Dakocytomakon (Dako). Images were acquired at ×1000 magnification using a Zeiss Axioplan, captured with a CCD camera (Photometrix sensys) and processed with Metamorph software. Single unbudded cells with one nucleus and mother cells with a small bud were designated as a G1/S cells. Mother cells with an equal sized daughter and nucleus at their budneck were designated as G2/M cells. For every time point, at least 50 cell-bodies were counted. Because of their poor growth, *rad52Δ* strain cell colonies from fresh spore were directly resuspended in water and observed using a visible light microscope. Comparisons between the cell sizes of mother and daughter cells were used to estimate the percentage of cells in G2/M phase.

**ChIP experiments.** ChIPs of MEC1–HA- and TEL1–HA-expressing strains were performed as described<sup>36</sup> using primers defined previously<sup>5</sup>.

**DNA analysis.** Telomere-PCR and subsequent telomere cloning and sequence analysis was performed as described previously<sup>13</sup> using oligonucleotides oT100 (5'-CTGTAGGGCTAAAGAACAGGG-3'), oT155 (5'-GGAAGTTCGGAATAGGAAGTTCAGACTCGAGAGATCCC-3') and oT157 (5'-ACCAAGTTGATGAGCAAGGG-3'). The oT100 oligonucleotide specifically amplifies long Y' telomeres<sup>10</sup>. Sequence reactions were performed by FASTER S. A. (Switzerland). To estimate the length of the pure TG<sub>1-3</sub> tract from the mobility of telomere-PCR, we first deducted the subtelomeric region and the oligonucleotide length and then applied a correction. The latter was estimated by comparing the mobility of telomere-PCR products with a Southern blot of the same DNA or by estimating the restriction fragment size after cloning or by direct sequencing of the resulting plasmids. The corrections applied were: oT155,  $y = 0.97 \times -83$ ; oT157,  $y = 0.87 \times -18.7$  and oT100,  $y = 0.95 \times -98$ , where  $\times$  is the median size of the telomere band in base pairs. These measurements were valid for telomere-PCR reactions performed at the same time and migrated in the same gel. Sequences of cloned telomeres are available on request.

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DOI: 10.1038/ncb1911



**Figure S1 a:** Two spores (#A and #B) of indicated genotype from experiment shown in Figure 2a were followed in 8 separate replicas of a serial spot assay. **b, d, f, g, i:** Serial spot assays of strains with indicated genotype that were used for viability plots shown in Figure 3 and 4. **c:** Viability curve obtained as described in Figure 2b from serial spots in Supplementary Figure 1b. **e:** Freshly germinated colonies grown from

spores of the indicated genotype were directly inspected under visible microscope to determine the cell cycle distribution. **h:** cells of indicated genotype at indicated passage were grown to exponential growth and treated as for Figure 2c to determine cell cycle stage. Abbreviations are: sp, spore colony; p1, passage 1; p2, passage 2; p3, passage 3; p4, passage 4.

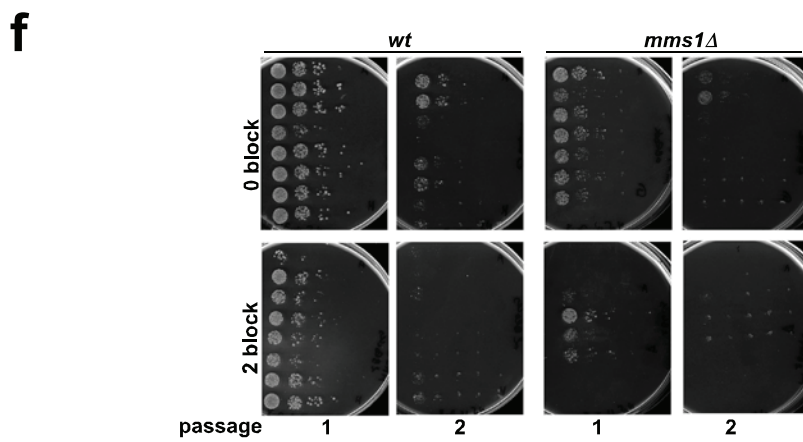
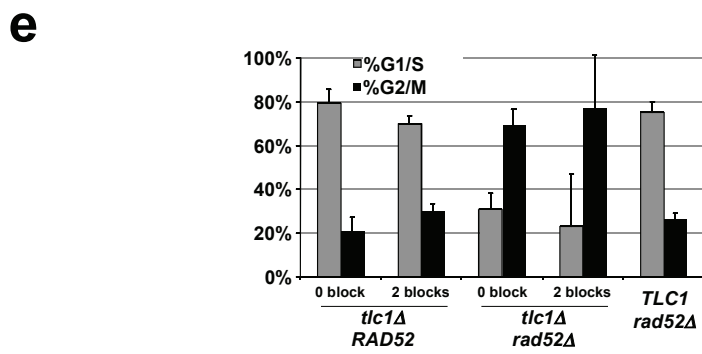
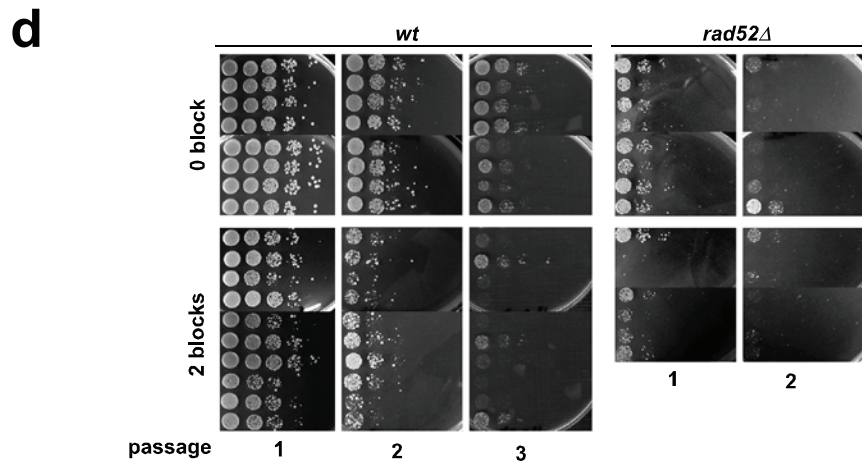
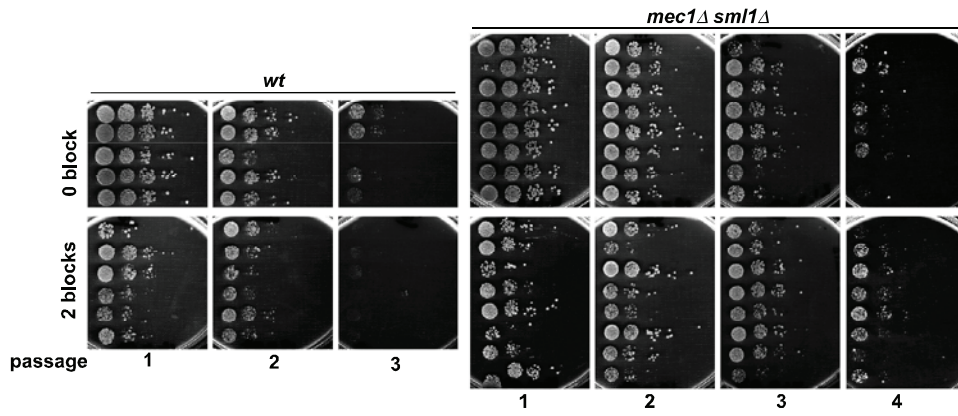
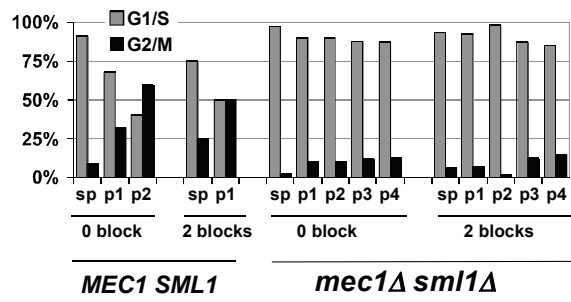


Figure S1 continued

**g**



**h**



**i**

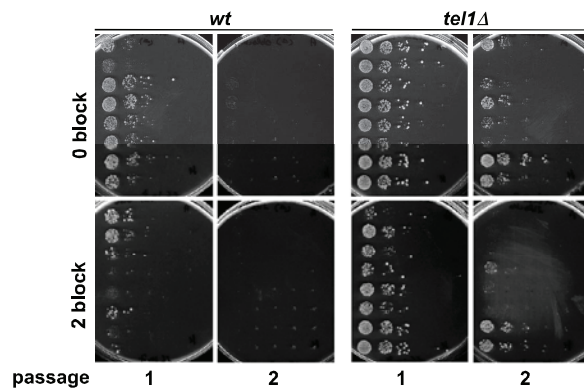


Figure S1 continued

**Table 1: p-values of the 2-tailed Wilcoxon rank sum test used to compare viabilities measured with the indicated strains.**

Set 1	Set 2	passage	p-value
<i>wt 0 Blocks n=8</i>	<i>wt 1 Block n=8</i>	1	0.003
<i>wt 0 Blocks n=8</i>	<i>wt 1 Block n=8</i>	2	0.002
<i>wt 0 Blocks n=8</i>	<i>wt 1 Block n=8</i>	3	0.030
<i>wt 0 Blocks n=8</i>	<i>wt 2 Blocks n=8</i>	1	0.001
<i>wt 0 Blocks n=8</i>	<i>wt 2 Blocks n=8</i>	2	0.001
<i>wt 0 Blocks n=8</i>	<i>wt 2 Blocks n=8</i>	3	0.054
<i>PTC2 0 Blocks n=8</i>	<i>wt 2 Blocks n=8</i>	1	0.001
<i>PTC2 0 Blocks n=8</i>	<i>wt 2 Blocks n=8</i>	2	0.048
<i>ptc2Δ 0 Blocks n=8</i>	<i>wt 2 Blocks n=8</i>	1	0.005
<i>ptc2Δ 0 Blocks n=8</i>	<i>wt 2 Blocks n=8</i>	2	0.010
<i>PTC2 0 Blocks n=8</i>	<i>ptc2Δ 0 Blocks n=8</i>	1	0.005
<i>PTC2 0 Blocks n=8</i>	<i>ptc2Δ 0 Blocks n=8</i>	2	0.574
<i>PTC2 2 Blocks n=8</i>	<i>ptc2Δ 2 Blocks n=8</i>	1	0.152
<i>RAD52 0 Blocks n=8</i>	<i>RAD52 2 Blocks n=10</i>	1	0.0002
<i>RAD52 0 Blocks n=8</i>	<i>RAD52 2 Blocks n=10</i>	2	< 0.0001
<i>RAD52 0 Blocks n=8</i>	<i>RAD52 2 Blocks n=10</i>	3	0.028
<i>rad52Δ 0 Blocks n=8</i>	<i>rad52Δ 2 Blocks n=7</i>	1	0.006
<i>rad52Δ 0 Blocks n=8</i>	<i>rad52Δ 2 Blocks n=7</i>	2	0.755
<i>RAD52 0 Blocks n=8</i>	<i>rad52Δ 0 Blocks n=8</i>	1	0.0002
<i>RAD52 0 Blocks n=8</i>	<i>rad52Δ 0 Blocks n=8</i>	2	0.001
<i>RAD52 2 Blocks n=10</i>	<i>rad52Δ 2 Blocks n=7</i>	1	0.001
<i>RAD52 2 Blocks n=10</i>	<i>rad52Δ 2 Blocks n=7</i>	2	0.001
<i>MMS1 0 Blocks n=8</i>	<i>MMS1 2 Blocks n=7</i>	1	0.152
<i>MMS1 0 Blocks n=8</i>	<i>MMS1 2 Blocks n=7</i>	2	0.050
<i>mms1Δ 0 Blocks n=7</i>	<i>mms1Δ 2 Blocks n=6</i>	1	0.022
<i>mms1Δ 0 Blocks n=7</i>	<i>mms1Δ 2 Blocks n=6</i>	2	0.713
<i>MMS1 0 Blocks n=8</i>	<i>mms1Δ 0 Blocks n=7</i>	1	0.002
<i>MMS1 0 Blocks n=8</i>	<i>mms1Δ 0 Blocks n=7</i>	2	0.017
<i>MMS1 2 Blocks n=7</i>	<i>mms1Δ 2 Blocks n=6</i>	1	0.002
<i>MMS1 2 Blocks n=7</i>	<i>mms1Δ 2 Blocks n=6</i>	2	0.044
<i>MEC1 SML1 0 Blocks n=5</i>	<i>MEC1 SML1 2 Blocks n=6</i>	1	0.004
<i>MEC1 SML1 0 Blocks n=5</i>	<i>MEC1 SML1 2 Blocks n=6</i>	2	0.052
<i>MEC1 SML1 0 Blocks n=5</i>	<i>MEC1 SML1 2 Blocks n=6</i>	3	0.026
<i>mec1Δ sml1Δ 0 Blocks n=8</i>	<i>mec1Δ sml1Δ 2 Blocks n=8</i>	1	0.001
<i>mec1Δ sml1Δ 0 Blocks n=8</i>	<i>mec1Δ sml1Δ 2 Blocks n=8</i>	2	0.328
<i>mec1Δ sml1Δ 0 Blocks n=8</i>	<i>mec1Δ sml1Δ 2 Blocks n=8</i>	3	0.442
<i>mec1Δ sml1Δ 0 Blocks n=8</i>	<i>mec1Δ sml1Δ 2 Blocks n=8</i>	4	0.161
<i>MEC1 SML1 0 Blocks n=5</i>	<i>mec1Δ sml1Δ 0 Blocks n=8</i>	1	0.943
<i>MEC1 SML1 0 Blocks n=5</i>	<i>mec1Δ sml1Δ 0 Blocks n=8</i>	2	0.171
<i>MEC1 SML1 0 Blocks n=5</i>	<i>mec1Δ sml1Δ 0 Blocks n=8</i>	3	0.019
<i>MEC1 SML1 2 Blocks n=6</i>	<i>mec1Δ sml1Δ 2 Blocks n=8</i>	1	0.950
<i>MEC1 SML1 2 Blocks n=6</i>	<i>mec1Δ sml1Δ 2 Blocks n=8</i>	2	0.108
<i>MEC1 SML1 2 Blocks n=6</i>	<i>mec1Δ sml1Δ 2 Blocks n=8</i>	3	0.002
<i>TEL1 0 Blocks n=8</i>	<i>TEL1 2 Blocks n=8</i>	1	0.010
<i>TEL1 0 Blocks n=8</i>	<i>TEL1 2 Blocks n=8</i>	2	0.282
<i>tel1Δ 0 Blocks n=8</i>	<i>tel1Δ 2 Blocks n=8</i>	1	0.007
<i>tel1Δ 0 Blocks n=8</i>	<i>tel1Δ 2 Blocks n=8</i>	2	0.234
<i>TEL1 0 Blocks n=8</i>	<i>tel1Δ 0 Blocks</i>	1	0.004
<i>TEL1 0 Blocks n=8</i>	<i>tel1Δ 0 Blocks</i>	2	0.002
<i>TEL1 2 Blocks n=8</i>	<i>tel1Δ 2 Blocks n=8</i>	1	0.010

## List of strains constructed in this work

yT136: *MATa/α ura3-1/ura3-1 trp1-1/trp1-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 tlc1::PralphaNat/TLC1 telVIIadh4::FRT-URA3-FRT-tel/telVIIadh4::FRT-URA3-FRT-tel*

yT137: *MATa/α ura3-1/ura3-1 trp1-1/trp1-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 tlc1::PralphaNat/TLC1 telVIIadh4::FRT-URA3-[tel270]-FRT-tel/telVIIadh4::FRT-URA3-[tel270]-FRT-tel*

yT138: *MATa/α ura3-1/ura3-1 trp1-1/trp1-1 leu2-3,112::Gal-FLP1-LEU2/leu2-3,112::Gal-FLP1-LEU2 his3-11,15/his3-11,15 can1-100/can1-100 ade2-1/ade2-1 tlc1::PralphaNat/TLC1 telVIIadh4::FRT-URA3-[tel270]x2-FRT-tel/telVIIadh4::FRT-URA3-[tel270]x2-FRT-tel*

yT208: yT136 *ptc2::KanMX4/PTC2*

yT210: yT138 *ptc2::KanMX4/PTC2*

yT143: yT136 *rad52::kanMX4/RAD52*

yT144: yT137 *rad52::kanMX4/RAD52*

yT145: yT138 *rad52::kanMX4/RAD52*

yT211: yT136 *mms1::kanMX4/MMS1*

yT212: yT138 *mms1::kanMX4/MMS1*

yT198: yT136 *mec1::KanMX2/MEC1 sml1::HIS/SML1*

yT199: yT138 *mec1::KanMX2/MEC1 sml1::HIS/SML1*

yT177: yT136 *MEC1-HA/MEC1 sml1::HIS/SML1*

yT179: yT138 *MEC1-HA/MEC1 sml1::HIS/SML1*

yT174: yT136 *tel1::HIS3/TEL1*

yT176: yT138 *tel1::HIS3/TEL1*

yT159: yT136 *TEL1-HA/TEL1*

yT161: yT138 *TEL1-HA/TEL1*

yT206: yT136 *mec1::KanMX2/MEC1 sml1::HIS/SML1 TEL1-HA/TEL1*

yT207: yT138 *mec1::KanMX2/MEC1 sml1::HIS/SML1 TEL1-HA/TEL1*