Telomerase-Null Survivor Screening Identifies Novel Telomere Recombination Regulators

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Abstract

Telomeres are protein–DNA structures found at the ends of linear chromosomes and are crucial for genome integrity. Telomeric DNA length is primarily maintained by the enzyme telomerase. Cells lacking telomerase will undergo senescence when telomeres become critically short. In Saccharomyces cerevisiae, a very small percentage of cells lacking telomerase can remain viable by lengthening telomeres via two distinct homologous recombination pathways. These “survivor” cells are classified as either Type I or Type II, with each class of survivor possessing distinct telomeric DNA structures and genetic requirements. To elucidate the regulatory pathways contributing to survivor generation, we knocked out the telomerase RNA gene TLC1 in 280 telomere-length-maintenance (TLM) gene mutants and examined telomere structures in post-senescent survivors. We uncovered new functional roles for 10 genes that affect the emerging ratio of Type I versus Type II survivors generation. We further verified that Pif1 helicase was required for Type I recombination and that the INO80 chromatin remodeling complex greatly affected the emerging frequency of Type I survivors. Finally, we found the Rad6-mediated ubiquitination pathway and the KEOPS complex were required for Type II recombination. Our data provide an independent line of evidence supporting the idea that these genes play important roles in telomere dynamics.

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Introduction

Telomeres are special DNA-protein structures found at the ends of eukaryotic chromosomes. Telomeres are crucial for genome integrity because they prevent chromosome ends from degradation or fusing with each other [1]. In budding yeast Saccharomyces cerevisiae, telomeric DNA consists of ~350 base pairs (bp) of TG1–3/C1–3 A repeats with a terminal single-stranded TG1–3 tract called a G-overhang [2]. Telomeric DNA can be maintained by either telomerase-mediated elongation or homologous recombination [3–5]. In budding yeast cells, the telomerase pathway supercedes the recombination pathway as the predominant mechanism of telomeric DNA elongation [6,7]. In telomerase-null cells, telomeric DNA is maintained via a recombination pathway termed “alternative lengthening of telomeres” (ALT) [10]. Approximately 85% of immortalized human tumor cells use telomerase to maintain telomeres while 15% apply the ALT mechanism to maintain telomeres [11].

In telomerase-null S. cerevisiae mutants, most cells undergo senescence after about 50–100 divisions when telomeres shorten to less than approximately 100 bp [7,12,13]. Surprisingly, a select few of these senescing cells are able to bypass the short telomere survival crisis through lengthening their telomeres via a Rad52-dependent recombination pathway [14]. These cells are called post-senescence survivors or “survivors” for short [14]. Survivors are categorized into two types: Type I and Type II, which possess different telomeric DNA structures and are defined by their dependence on Rad51 or Rad50 respectively [15]. Type I survivors exhibit highly amplified subtelomeric Y’ elements and short terminal telomeric TG tracts. The formation of Type I survivors depends on the canonical homologous recombination proteins Rad51, Rad54, Rad55 and Rad57 [14]. On the other hand, Type II survivors have long heterogeneous terminal telomeric TG tracts generated by recombination, and their formation depends on the Mre11-Rad50-Xrs2 (MRX) complex and Rad59 [14]. Type II survivors resemble the ALT cells observed in mammals [5]. In S. cerevisiae, about 90% of survivors generated on solid medium are categorized as Type I, while 10% are Type II. Nevertheless, Type II survivors grow at faster rates than Type I survivors, eventually overtaking their counterparts in liquid-grown cultures [14].
Author Summary

Homologous recombination is a means for an organism or a cell to repair damaged DNA in its genome. Eukaryotic chromosomes have a linear configuration with two ends that are special DNA–protein structures called telomeres. Telomeres can be recognized by the cell as DNA double-strand breaks and subjected to repair by homologous recombination. In the baker’s yeast Saccharomyces cerevisiae, cells that lack the enzyme telomerase, which is the primary factor responsible for telomeric DNA elongation, are able to escape senescence and cell death when telomeres undergo repair via homologous recombination. In this study, we have performed genetic screens to identify genes that affect telomeric DNA recombination. By examining the telomere structures in 280 mutants, each of which lacks both a telomere-length-maintenance gene and telomerase RNA gene, we identified 32 genes that were not previously known to be involved in telomere recombination. These genes have functions in a variety of cellular processes, and our work provides new insights into the regulation of telomere recombination in the absence of telomerase.

In addition to the proteins in the Rad52 epistasis group, which are well-defined in the canonical survivor formation pathways, other genes involved in survivor formation have sporadically been identified. For example, SGS1, MEC1/TEL1, MDT1, DEF1, CLB2 and SUA5 are required for the generation of Type II survivors, while RIF1 and RIF2 have strong influences toward Type I survivor emerging frequency [16–22]. Notably, some of the genes mentioned above appear to contribute to both survivor generation and telomere length regulation. Deletion of RIF1 or RIF2 causes telomere shortening, while deletion of MRE11, RAD50, XRS2, TEL1, DEF1 or SUA5 results in telomere shortening [16,23–25]. These observations suggest that genes involved in telomere recombination pathways and telomere length regulation are in some way linked. So far, there have been 251 telomere length maintenance (TLM) genes identified by genome-wide screens [23,26] and other studies [16,27–34]. Furthermore, 29 additional genes previously miss-classified as essential genes in the Saccharomyces genome deletion project have now officially been implicated as TLM genes [24]. In this study we deleted the TLM gene encoding the RNA template subunit of telomerase in each of these 280 TLM mutants. We then examined the survivor types that arose and in doing so we were able to identify novel regulators that contribute to telomere recombination. The genes we characterized as telomere recombination regulators may also affect general DNA recombination at other genomic loci.

Results

Screening of TLM gene deletion library on solid medium identifies genes affecting the emerging ratio of Type I versus Type II survivors

To search for genes affecting survivor formation, we knocked out the RNA component of telomerase TLM1 in 280 haploid TLM mutants reported to have longer or shorter telomeres than the wild-type strain [23,24,26] (Table S1). Knocking out TLM1 in most TLM mutants is typically achieved by transformation of an integrating plasmid but for some strains with extremely short telomeres or severe growth defects, recovering a TLM1 deletion clone using this approach was not possible. For such cases, we mated tlc1Δ mutant (BY4741 background) with tlmΔ mutants (BY4742 background) to generate heterozygous diploid strains, and then performed tetrad dissection to obtain haploid mutants lacking both TLM1 and TLM genes (Table S1).

After a telomerase-null tlmΔ mutant library was established, each mutant was passaged repeatedly on solid plates to screen for genes that might affect Type I survivor formation. Most of the mutant cells underwent senescence but a small percentage of cells were able to overcome crisis and become survivors [5]. Genomic DNA was extracted from each survival isolate, digested with the XhoI restriction enzyme, and analyzed by Southern blot with a TG probe to determine if the cells were Type I or Type II survivors (Figure 1A) (see Materials and Methods). In the first round of screening for genes affecting Type I survivor formation, we passaged two independent senescing colonies from each mutant on solid plates to obtain survivors. Because the emerging frequency of Type I survivors (~90%) is much higher than that of Type II survivors (~10%), most double mutants passed on a solid plate, like the tlc1Δ single mutant, turned out to be Type I survivors [5]. However, if both of the two colonies picked from a single mutant strain had telomere structures consistent with that of Type II survivors, it was concluded that the gene missing in this Type II strain might contribute to Type I survivor generation and should be analyzed further. For each tlc1Δ tlmΔ mutant selected in this first round of rough screening, eight single colonies were passaged on solid plates in the second round of screening until survivors arose. When more than four colonies became Type II survivors, this TLM gene was subjected to a third round of screening in which fifty colonies of the tlc1Δ tlmΔ mutant were passaged again on solid plates. From these fifty colonies at least forty colonies typically generated survivors that could be examined. The emerging frequency of Type II survivors in each strain was then calculated (Table 1). Using this screening approach we identified eleven mutants in which the emerging frequencies of Type II survivors was elevated significantly (Table 1). Among these eleven genes, RIF1 and RIF2 deletion in telomerase-null tlc1Δ mutant generated Type II frequencies of 52.2% and 85.7% respectively (Table 1, the column of “Deleting TLC1 in tlm mutants”), percentages which are consistent with a prior study performed by Teng et al. [22]. The other nine genes that affected survivor formation have never before been reported to have such a function. The Type II emerging frequencies in these nine mutants ranged from 45.7% to 93.6% (Table 1, the column of “Deleting TLC1 in tlm mutants”) and were significantly elevated compared to that of the tlc1Δ cells, which had a Type II emerging frequency of 4% (Figure 1B). In contrast with the eleven genes that affected Type I survivor generation, the RIFI, helicase gene [35,36], appeared to be essential for Type I survivor generation (discussed later).

Very recently, Chang et al. showed that the long telomeres in rif1Δ tlc1Δ and rif2Δ tlc1Δ mutants were preferentially extended by a recombination pathway and senescent cells with long telomeres were more efficient at bypassing senescence via the Type II survivor pathway [37]. These led Chang et al. to propose that rif1Δ tlc1Δ and rif2Δ tlc1Δ mutants affect the ratio of survivor types by altering telomere length at the point of senescence [37]. In order to examine the idea that telomere length affects the type of survivor generated, we generated eleven TLM1/tlc1Δ TLM1/tlmΔ diploid strains and performed tetrad dissections to obtain tlc1Δ single and tlc1Δ tlmΔ double mutants (Table 1, the column of “Spore from tetrad dissection”). Because the ino409Δ tlc1Δ double mutant used in the previous experiments was obtained from tetrad dissection, it was not included in this experiment. Fifty senescing clones of the other ten mutant strains, including tlc1Δ single mutants from each diploid mutant, were streaked on plates until...
survivors arose. Telomere structures of the survivors generated on plates were examined by Southern blot (Figure S1). A representative Southern blot result of \( \text{rpa14}\Delta \text{tlc1}\Delta \) mutant is shown in Figure 1C. The results of these experiments are summarized below and are listed in the column of “Spore from tetrad dissection” in Table 1. The frequency of Type II survivor formation in the \( \text{sap30}\Delta \text{tlc1}\Delta \), \( \text{rpa14}\Delta \text{tlc1}\Delta \), \( \text{rrp8}\Delta \text{tlc1}\Delta \) and \( \text{gup1}\Delta \text{tlc1}\Delta \) double mutants was decreased when compared to that of the corresponding double mutant that had not been through sporogenesis. The frequency of Type II survivor formation in the \( \text{rpb9}\Delta \text{tlc1}\Delta \) or \( \text{rps16b}\Delta \text{tlc1}\Delta \) double mutants was increased when compared to that of the corresponding double mutant that had not been through sporogenesis. The frequency of Type II survivor formation in the \( \text{rif1}\Delta \text{tlc1}\Delta \), \( \text{rif2}\Delta \text{tlc1}\Delta \) and \( \text{soh1}\Delta \text{tlc1}\Delta \) double mutants did not change significantly. Recovery of the \( \text{ies3}\Delta \text{tlc1}\Delta \) double mutant from sporogenesis was not successful. We also examined telomere length around the time of survivor formation and found that similar to the \( \text{rif1}\Delta \text{tlc1}\Delta \) and \( \text{rif2}\Delta \text{tlc1}\Delta \) mutants, the critical telomere length in \( \text{gap30}\Delta \text{tlc1}\Delta \) and \( \text{ans50}\Delta \text{tlc1}\Delta \) mutants was about 50 bp longer than those in \( \text{tlc1}\Delta \) single mutants from the same crosses (Figure S2). However, in \( \text{sub1}\Delta \text{tlc1}\Delta \) and \( \text{rph3}\Delta \text{tlc1}\Delta \) mutants, the critical telomere lengths were about 30 bp shorter than those in \( \text{tlc1}\Delta \) mutants from the same crosses (Figure S2). Additionally, in the \( \text{rps16b}\Delta \text{tlc1}\Delta \), \( \text{gap30}\Delta \text{tlc1}\Delta \) and \( \text{rph3}\Delta \text{tlc1}\Delta \) mutants, the critical telomere lengths were slightly longer (<30 bp) than those in \( \text{tlc1}\Delta \) mutants from the same crosses (Figure S2). In the \( \text{rpa14}\Delta \text{tlc1}\Delta \) mutant, the critical telomere length was similar to that in \( \text{tlc1}\Delta \) mutant from the same cross (Figure S2). Our data support the idea put forth by Cheng et al. that telomere length affects survivor formation [37]. Our data also show the frequency of Type II emergence in the nine mutants we identified ranged from 44.7% to 90.5%, which was much higher.

Figure 1. Identification of genes affecting the emerging ratio of Type I versus Type II survivors. (A) Schematic illustration of the screening procedures for genes that affect the emerging ratio of Type I vs Type II survivors (refer to details in main text). The \( \text{tlc1}\Delta \) single mutants and the \( \text{rpa14}\Delta \text{tlc1}\Delta \) double mutants were generated through tetrad dissection from heterozygous diploids with one copy of \( \text{RPA14} \) and \( \text{TLCl} \) deleted. Fifty independent colonies of each mutant were randomly selected and passaged on plates, and the telomere structures of survivors were examined by Southern blot using a TG probe. (B and C) The Southern blot analysis of survivor types in \( \text{tlc1}\Delta \) strain (control) (B) and the \( \text{rpa14}\Delta \text{tlc1}\Delta \) mutant (C). The asterisks (*) in (B) indicate Type II survivors. The triangles (\( \triangledown \)) in (C) indicate Type I survivors.

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than the Type II emerging frequencies of less than 10% that were usually observed in \textit{tlc1}Δ cells (Table 1 and Figure S1) [5].



**Table 1.** List of \textit{S. cerevisiae} TLM genes affecting Type I versus II survivor ratio in \textit{tlc1}Δ cells.

| Gene | Tel Length | Type II Frequency | Function (Annotation from \textit{Saccharomyces cerevisiae} Genome Database) |
|------|-------------|-------------------|------------------------------------------------------------------------------|
|      | Deleting \textit{TLC1} in \textit{tlm} mutants | Spores from tetrad dissection |                                  |
| **Telomere capping or maintenance** | | | |
| \textit{RIF1} | L | 52.2% (24/46)a | 50% (24/48) | Telomeric protein, binds to Rap1 |
| \textit{RIF2} | L | 85.7% (42/49) | 85% (34/40) | Telomeric protein, binds to Rap1 |
| **Chromatin remodeling or modification** | | | |
| \textit{IES3} | L | 85.4% (41/48) | NDb | Subunit of \textit{INO80} chromatin remodeling complex |
| \textit{INO80} | S | NDb | 72% (36/50) | Subunit of \textit{INO80} chromatin remodeling complex |
| **DNA-dependent Transcription** | | | |
| \textit{RPA14} | L | 73.5% (36/49) | 59.6% (28/47) | RNA polymerase I subunit A14 |
| \textit{RBP9} | S | 64.6% (31/48) | 90.5% (38/42) | RNA polymerase II subunit B12.6 |
| \textit{SOH1} | S | 48% (24/50) | 46.3% (19/41) | Subunit of the RNA pol II mediator complex |
| **rRNA processing** | | | |
| \textit{RRP8} | L | 93.6% (44/47) | 50% (28/50) | Methyltransferase, pre-rRNA cleavage at site A2 |
| **Structural constituent of ribosome** | | | |
| \textit{RPS16B} | L | 45.7% (21/46) | 53.2% (25/47) | Component of the small (40S) ribosomal subunit |
| **Transport & membrane** | | | |
| \textit{GUP1} | S | 62.5% (30/48) | 51.1% (23/45) | Plasma membrane protein in glycerol uptake |

aNumbers in parenthesis shown as (a/b): “a” stands for the number of colonies which turned to be Type II survivors among 50 colonies; “b” stands for the number of post-senescence colonies which became survivors among 50 colonies.
bSpores were not generated in isogenic \textit{tlc1}Δ \textit{ies3}Δ double mutant.

\textit{INO80} chromatin remodeling complex affects the emerging frequency of Type I survivor generation

The \textit{INO80} complex is one of the ATP-dependent chromatin remodeling complexes that can move or evict nucleosomes, thereby changing chromatin structure and affecting the accessibility of DNA to other factors [38]. The yeast \textit{INO80} complex contains multiple subunits, including five essential and ten (\textit{Ino80}, \textit{Ies1}, \textit{Ies2}, \textit{Ies3}, \textit{Ies4}, \textit{Ies5}, \textit{Ies6}, \textit{Taf14}, \textit{Arp8} and \textit{Nhp10}) non-essential subunits [38]. A recent study has shown that \textit{Ies5} interacts with the telomerase component Est1 [34]. In \textit{est1}Δ cells, deleting \textit{IES3} or \textit{ARP8} caused a delay of survivor generation in liquid culturing [34], suggesting that the \textit{INO80} complex affects telomere recombination. In our survivor screening we noted that two subunits in the \textit{INO80} complex, \textit{Ino80} and \textit{Ies3}, significantly affected the generation of Type I survivors (Table 1). When passed on solid medium, the \textit{ino80}Δ \textit{tlc1}Δ and \textit{ies3}Δ \textit{tlc1}Δ mutants produced Type II survivors at frequencies of 70% and 85.4% respectively (Figure 2A and 2B), which were significantly elevated in comparison with the 8.3% we observed in \textit{tlc1}Δ cells (Figure 2B and Figure S3A). These results suggested that the \textit{INO80} complex may be required for efficient Type I survivor formation. To examine this possibility further we examined the impact of deleting each of the other four non-essential subunits of the \textit{INO80} complex on the efficiency of Type I survivor formation in \textit{tlc1}Δ cells. The Southern blot results revealed that the deletion of each of the non-essential \textit{INO80} subunits \textit{IES1}, \textit{IES4}, \textit{IES5} and \textit{NHP10} led to the generation of more Type II than Type I survivors (Figure S3). The frequency of Type II emergence in each of these mutants in \textit{tlc1}Δ cells was above 60% (Figure 2B), which was much higher than that of the \textit{tlc1}Δ single mutant. These results indicate that the \textit{INO80} complex greatly influences the emerging ratio of Type I vs Type II survivors.

\textit{Pif1} is required for Type I recombination

\textit{PFI1} is a non-essential gene which encodes a 5’ to 3’ DNA and RNA/RNA helicase in \textit{S. cerevisiae} [36,39]. Previous studies have demonstrated that \textit{Pif1} can be translated from different start sites and has two forms which are localized to either the mitochondria or the nucleus [35,40]. In the mitochondria \textit{Pif1} affects recombination of mitochondrial DNA (mtDNA) and plays an important role in maintaining mtDNA stability [41–43]. In the nucleus, \textit{Pif1} inhibits telomere lengthening by removing telomerase from telomeric DNA [35,44] and participates in Okazaki fragment maturation [45,46] and ribosomal DNA replication [47]. Additionally, \textit{Pif1} is able to unwind G-quadruplex structures \textit{in vitro} [48], and likely acts on these structures \textit{in vivo} as well [48,49].

In our primary screening the \textit{pfi1}Δ \textit{tlc1}Δ double mutant had difficulties generating survivors on solid medium, and as a result most clones died out during sequential streaks. The \textit{pfi1}Δ \textit{tlc1}Δ clones that overcame senescence on solid medium showed a Type II survivor pattern (Figure 3A and 3B), suggesting that \textit{Pif1} promotes Type I survivor formation. To further validate the role of \textit{Pif1} in Type I survivor generation, we streaked fifty independent \textit{pfi1}Δ \textit{tlc1}Δ colonies on plates. We noted that forty
post-senescence colonies (80%) died during the sequential streaks, indicating that deletion of PIF1 in telomerase-null strains inhibits the creation of post-senescence survivors. The other 10 colonies also underwent senescence, but were able to generate survivors at the 7th streaking. Cells at this stage were harvested, and their telomeres were examined by Southern blot assay (Figure 3C). Only two colonies (4%), which grew at a normal rate, gave rise to type II survivors (Figure 3C and 3D), indicating that type II survivors can indeed form in the absence of Pif1. Interestingly, eight colonies (16%) of extremely slow growing survivors showed distinct patterns of telomeric DNA without either long heterogeneous TG tracts or substantial Y' amplification (Figure 3C and 3D), suggesting that a new type of survivor emerged in pif1Δ tlc1Δ post-senescence cells. In these cells the terminal TG tracts seemed to be even shorter than that in Type I survivors but were unexpectedly maintained during subsequent passages. This abnormality of telomeric DNA was also observed by Dewar et al. [50]. Nevertheless our results suggested that Pif1 is required for Type I survivor generation. To confirm this further, since RAD50 and RAD51 are respectively required for Type II and Type I survivor formation, we checked whether survivors could form in either a rad50Δ pif1Δ tlc1Δ or a rad51Δ pif1Δ tlc1Δ triple mutant. The isogenic rad50Δ pif1Δ tlc1Δ or rad51Δ pif1Δ tlc1Δ spores were dissected and serially passaged in liquid culture. As expected, two spores of the rad50Δ pif1Δ tlc1Δ triple mutant underwent senescence gradually and virtually died out at the 9th or 11th passage (Figure 3E). A Southern blot analysis revealed that the telomere structures of these post-senescence survivors were very similar to those of the pif1Δ tlc1Δ mutant (Figure 3H). We therefore concluded that Pif1’s helicase activity plays a key role in telomeric DNA recombination.

Helicases are nucleic acid-dependent ATP-ases that are capable of unwinding DNA or RNA duplex substrates and play important roles in almost every cellular process including DNA replication and repair, transcription, translation, RNA processing and so on [51,52]. In S. cerevisiae, there are 132 open-reading-frames that encode helicase or helicase-like proteins [35]. Thirteen of them have been shown to have DNA helicase activity. We knocked out TLC1 in each of these thirteen DNA helicase gene mutants (Figure S4) and carried out survivor screenings to investigate if these genes affect Type I or Type II survivor generation. In contrast with

| Strain                  | Deleting TLC1 directly in tim mutants | Spores from tetrad dissection |
|-------------------------|---------------------------------------|------------------------------|
| tlc1Δ                   | 8.3% (4/48)                           | 8.5% (4/47)                  |
| ino80Δ tlc1Δ            | ND                                    | 70% (35/50)                  |
| ies1Δ tlc1Δ             | 60% (6/10)                            | 64% (32/50)                  |
| ies3Δ tlc1Δ             | 85.4% (41/48)                         | ND                           |
| ies4Δ tlc1Δ             | 81.6% (40/49)                         | 80% (40/50)                  |
| ies5Δ tlc1Δ             | 80% (8/10)                            | 91.8% (45/49)                |
| nhp10Δ tlc1Δ            | 91.8% (45/49)                         | 71.4% (35/49)                |

Figure 2. The effect of the Ino80 complex on survivor formation. (A) Fifty independent survivor colonies of the ino80Δ tlc1Δ mutant, which was generated from INO80/ino80Δ TLC1/tlc1Δ diploid mutant, were randomly picked and their genomic DNA was isolated for Southern blot assay using a TG1–3 probe. The black triangles indicate Type I survivors. (B) Chart of Type II survivor frequencies in the mutants of Ino80 complex subunits. ND: not done (see Table 1).
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PIF1, the other twelve DNA helicase genes and TLC1 double deletion mutants generated Type I survivors on solid medium, indicating that they are not essential for Type I survivor formation (Figure S4A). In liquid medium, sgs1Δ tlc1Δ cells generated Type I survivors, while the other twelve DNA helicase genes and TLC1 double deletion mutants generated Type II survivors after passing 12 times (about 200 population doublings) (Figure S4B). This result is consistent with a previous report which shows Sgs1 helicase is required for Type II survivor formation [53]. We obtained the pif1Δ sgs1Δ tlc1Δ triple mutant dissected from the heterozygous PIF1/pif1Δ SGS1/sgs1Δ TLC1/tlc1Δ diploid mutant. The pif1Δ sgs1Δ tlc1Δ mutant was cultured in liquid medium and no survivors were recovered (Figure 3E). It was therefore concluded that Pif1 and Sgs1 may define the Type I and Type II survivor formation pathways respectively.

Screening of TLM gene deletion library in liquid medium identifies genes affecting Type II survivor formation

In order to screen for genes that might affect Type II survivor formation, we grew the 280 telomerase-null tlmΔ mutants serially in liquid medium to generate survivors (Figure 4A) [3]. If Type II survivors arise, they eventually out-compete their Type I counterparts in liquid culture because of their aforementioned growth advantage [5]. There were, however, some strains that lacked the genes required for Type II survivor formation, and thus generated only Type I survivors. The viability of these senescing mutants was recorded during passages and survivor cells were harvested at the end of serial culturing. The genomic DNA of the liquid-cultured cells was isolated and subjected to Southern blot with a telomeric TG13 probe. Twenty-four tlc1Δ tlnΔ double mutants formed Type I survivors, suggesting these twenty-four genes were required for Type II survivor formation (Figure 4B and Table 2). To further confirm the Type I phenotypes of these mutants, we used a Y′ probe and performed Southern blot hybridization to examine the DNA structure. The results clearly showed significant amplification of Y′-elements, a characteristic typical of Type I survivors (Figure 4C).

Among these twenty-four genes, twenty-two had never before been identified for their involvement in Type II survivor formation (Table 2). The two genes identified in our screening that have been previously reported to maintain such a function include SUA5 and DEFI [16,18]. It is important to note that survivors generated in tlc1Δ yka70Δ or tlc1Δ yka80A cells exhibited distinct telomeric DNA patterns that differed from classical Type I and Type II survivor structures (Figure 4B and 4C, left panels) [54,55]. Moreover, both tlc1Δ yka70Δ and tlc1Δ yka80A mutant cells exhibited more rapid senescence and became survivors as soon as the telomeric DNA from germinating spores could be examined, observations which are consistent with earlier reports [55,56]. The results of the yka70Δ and yka80A mutants were presented in this section with the other mutants which displayed Type I survivors because survivor generation in tlc1Δ yka70Δ and tlc1Δ yka80A mutants is more dependent upon RAD51 than RAD50 [54].

Type II survivor formation involves the Rad6-Bre1 pathway

As mentioned above, we identified twenty-two genes not previously known to be required for Type II survivor formation (Table 2). RAD6 remains of particular interest as previous studies have shown that RAD6 plays important roles in recombinational repair [57]. Rad6 is an E2 ubiquitin-conjugating enzyme and it interacts with three E3 ubiquitin ligases (Bre1, Rad18 and Ubr1) known to be involved in different DNA repair pathways [58,59]. Rad6 and Bre1 are responsible for H2B-K123 ubiquitination, which is required for H3-K4 methylation [60]. Rad6 and Rad18 are involved in post-replication repair via their role in ubiquitination of PCNA [61]. Rad6 and Ubr1 have been linked to DNA repair through their function in degradation of cohesin [62]. Our Southern blot analysis showed that rad6Δ tlc1Δ double mutant cells in liquid culture generated only Type I survivors (Figure 5A), suggesting that Rad6 is required for Type II survivor formation. To validate this result, we knocked out RAD51, which is required for Type I survivor formation, in the rad6Δ tlc1Δ cells. All four clones of the rad6Δ rad51Δ tlc1Δ mutant underwent senescence and were unable to generate survivors (Figure S5B), confirming that Rad6 is required for Type II survivor formation. In order to determine the downstream pathways utilized by Rad6 during Type II survivor generation we constructed the heterozygous diploid strain of TLC1/tlc1Δ RAD51/rad50Δ BRE1/ bre1Δ ubr1Δ ubr1Δ. The isogenic haploid tlc1Δ ubr1Δ mutants displayed no obvious amplification of Y′-subtelomeric elements whereas the tlc1Δ bre1Δ tlc1Δ bre1Δ ubr1Δ and tlc1Δ bre1Δ rad18Δ survivors displayed no obvious amplification of Y′-subtelomeric elements whereas the tlc1Δ bre1Δ tlc1Δ bre1Δ ubr1Δ and tlc1Δ bre1Δ rad18Δ survivors that lacked the BRE1 gene displayed significant Y′-element amplification (Figure 5D). These data suggest Bre1 plays an even more positive regulatory role in Type II survivor generation than Ubr1 and Rad18. Interestingly, the tlc1Δ rad18Δ bre1Δ ubr1Δ mutant cells only allowed the development of Type I survivors. These results indicate that Rad6 functions through its
downstream pathways and most importantly Bre1 to promote Type II survivor formation.

The KEOPS complex is required for Type II recombination

In addition to RAD6, CGI121 and KAE1 were also identified during our liquid-culture screen as contributing to Type II survivor formation (Table 2). Cgi121 and Kae1 belong to the KEOPS complex, which is evolutionarily conserved from archaea to mammals [63]. In S. cerevisiae, the KEOPS complex consists of five subunits (Cgi121, Bud32, Kae1, Gon7 and Pcc1) and plays multiple roles in transcription, tRNA modification (t6A), chromosome segregation and telomere uncapping-elongation [29,64–66]. The deletion mutants of BUD32 and GON7 were in our original TLM library but in our initial screening the severe growth defects of the bud32Δ and gon7Δ haploid strain made it impossible for us to knockout TLC1. PCC1 was not in the 280 TLM gene list, and therefore was not covered in our initial screening. In order to determine whether Bud32, Gon7 and Pcc1 were also involved in telomere recombination, we constructed the heterozygous diploid mutants in which one copy of TLC1 and BUD32, GON7 or PCC1 were deleted. The double mutants of bud32Δ tlc1Δ, gon7Δ tlc1Δ and pcc1Δ tlc1Δ were obtained from tetrad dissection and were serially passaged in liquid medium. All the survivors displayed Type I patterns of Y’ amplification (Figure 6A), indicating that Type II recombination could not take place in the absence of Bud32, Gon7 or Pcc1.

To further confirm the critical role the KEOPS complex plays in telomere recombination, we tested whether survivor formation in cgi121Δ tlc1Δ cells would be affected in the absence of RAD51.

Figure 4. Southern blot analyses of Type I survivors generated in tlmΔ tlc1Δ mutants in liquid culture. (A) Schematic illustration of the screening procedures for genes that affect Type II survivor formation (refer to details in main text). (B and C) Southern blot analyses of survivor types in the tlc1Δ strain (Type I and Type II serves as controls) and twenty-six tlmΔ tlc1Δ double mutants using a TG probe (B) and a Y’ probe (C). doi:10.1371/journal.pgen.1003208.g004
RAD50. Unfortunately, we could not examine the genetic interaction between RAD51 or RAD50 and the other four KEOPS subunits in telomere recombination because the bud32Δ, kae1Δ, gon7Δ and pec1Δ mutants all exhibited severe growth defects. Therefore we focused on CGI121 by generating a heterozygous diploid strain in which one copy of TLC1, CGI121 and RAD51 (or RAD50) was deleted. The isogenic strains of single, double and triple mutants were derived from tetrad dissection and serially cultured in liquid medium. The cgi121Δtlc1Δrad51Δ triple mutant died out rapidly, while other tlc1Δ mutants were able to recover robust growth when survivors arose (Figure 6B). Consistently, the cgi121Δtlc1Δrad50Δ triple mutant was able to bypass the senescence crisis by generating Type I survivors (Figure 6C and 6D). These results support the conclusion that CGI121 and likely the entire KEOPS complex is required for Type II recombination.

Previous studies have shown that Kae1 has ATP-binding activity and Bud32 acts as a protein kinase and the activities of both of these gene products appear to be essential for all the roles played by the KEOPS complex [29,63,66,67]. Based on the previous biochemical and structural analyses of Kae1 and Bud32 [63], we constructed kae1Δtale1Δ, tlc1Δk52Δtlc1Δ and bud32Δn166Δtlc1Δ double mutant strains in which the Kae1 ATP-binding site and the Bud32 kinase catalytic sites were mutated. These mutants were unable to generate Type II survivors, but rather exclusively developed Type I survivors when cultured in liquid medium (Figure 6E and 6F). Likewise, the kae1Δcae1Δtale1Δ mutants, which no longer maintain an interaction between Kae1 and Bud32, also displayed a defect in Type II survivor generation (Figure 6E). These data indicate that both the Kae1-Bud32 interaction and their biochemical activities were indispensable for Type II telomere recombination. We therefore concluded that the whole KEOPS complex was necessary for Type II recombination.

### Table 2. List of S. cerevisiae TLM genes required for Type II survivor formation.

| Gene      | Tel Length | Function (Annotation from *Saccharomyces cerevisiae* Genome Database)                                      |
|-----------|------------|--------------------------------------------------------------------------------------------------------|
| CGI121    | S          | KEOPS complex                                                                                         |
| KAE1      | S          | KEOPS complex                                                                                         |
| SUA5      | S          | Telomeric ssDNA-binding, t6A modification (tRNA)                                                       |
| DEF1      | S          | Interacted with Rrm3p, RNAPII degradation factor                                                      |
| NMD2      | S          | Nonsense-mediated decay                                                                                |
| UPF3      | S          | Nonsense-mediated decay                                                                                |
| EBS1      | S          | NMD; inhibition of translation; EST1 homologue                                                        |
| NAM7      | S          | NMD; ATP-dependent RNA helicase                                                                       |
| CBC2      | L          | Component of the spliceosomal commitment complex                                                      |
| RAD6      | S          | E2 ubiquitin-conjugating enzyme in DNA repair                                                         |
| SLX8      | L          | 5x5-5x8 substrate-specific ubiquitin ligase complex                                                   |
| XRN1      | S          | Conserved 5’-3’ exonuclease component in mRNA decay                                                    |
| RRP17     |            | Exonuclease for 5’ end processing of pre-60S ribosomal RNA                                             |
| RPL13B    | S          | Component of the large (60S) ribosomal subunit                                                        |
| CDH1      | L          | Cell-cycle regulated activator of APC                                                                |
| MET7      | S          | Poly/polyglutamate synthetase                                                                       |
| ATM1      | S          | Mitochondrial inner membrane ATP-binding cassette transporter                                          |
| CYR1      | L          | Adenylate cyclase                                                                                     |
| MTR10     | S          | Nuclear import receptor                                                                               |
| LSG1      | S          | Putative GTPase; required for Nmd3p release from 60S subunits                                          |
| YPL142C   | S          | Dubious ORF                                                                                           |
| YDR396W   | S          | Dubious ORF                                                                                           |
| YDR413C   | S          | Dubious ORF                                                                                           |
| YGL069C   | S          | Dubious ORF                                                                                           |

Some TLM genes involved in telomere recombination also affect DNA recombination in general

Previously, several labs performed genome-wide screens searching for genes that affect DNA repair and/or recombination and...
dozens of genes were documented [68–71]. In this study we have identified ten genes which affect Type I telomere recombination and twenty-two genes which affect Type II telomere recombination. Fifteen of these genes have already been reported to have potential roles in general DNA repair and/or recombination (Table S2). In order to determine whether the other seventeen genes also play roles in general DNA repair/recombination, we performed three assays used previously [72,73] to examine relative levels of inter-chromosomal homologous recombination (Figure 7A) and intra-chromosomal homologous recombination in haploid (Figure 7B) and diploid strains (Figure 7C). Each assay detected genomic gene conversion events through the recovery of an intact \textit{LEU2} marker by the integration of two separated fragments (Figure 7A–7C, upper panels). Ten of the seventeen mutants we tested exhibited an extremely slow growth phenotype and were not viable for testing using the general recombination assays. The remaining seven mutants (\textit{nam7}Δ, \textit{eps1}Δ, \textit{upf3}Δ, \textit{rmd2}Δ, \textit{tsp16}Δ, \textit{soh1}Δ and \textit{cig121}Δ) showed decreased activities in inter- or intra-chromosomal homologous recombination (Figure 7A–7C). Therefore it is likely that these seven genes participate in telomere recombination as well as recombination at other genomic loci.

The INO80 complex, Pif1, and Rad6 affect telomere recombination through break-induced-replication mechanism

Break-induced-replication (BIR) only requires one free DNA end to take place and it has been proposed to be the principal mechanism for telomere recombination and survivor generation [14]. To examine whether the INO80 complex, Pif1, Rad6 and the KEOPS complex participate in telomere recombination via...
Figure 6. Each subunit of the KEOPS complex is essential for Type II recombination. (A) Southern blot of the genomic DNA of the survivors generated by serial liquid culturing of \textit{cgi121} \textit{tlc1}, \textit{pcc1} \textit{tlc1}, \textit{gon7} \textit{tlc1}, \textit{bud32} \textit{tlc1}, and \textit{kae1} \textit{tlc1} mutants. (B) Liquid culture cell viability analyses of sibling spores generated in the diploid \textit{CGI121/cgi121} \textit{TLC1/tlc1} \textit{RAD51/rad51} strain. The \textit{cgi121} \textit{tlc1} \textit{rad51} triple mutant died out at the 7th passage. (C) Cell viability analysis of sibling spores generated in the diploid \textit{CGI121/cgi121} \textit{TLC1/tlc1} \textit{RAD50/rad50} strain. Two spores were analyzed in parallel and the results were similar. (D) Southern blot analysis of telomere DNA in survivors generated by serial liquid culturing of the \textit{cgi121} \textit{tlc1} \textit{rad50} triple mutant. (E) and (F) Southern blot analyses of telomere DNA in survivors generated by serial liquid culturing of the \textit{tlc1} \textit{kae1} (E213R), \textit{tlc1} \textit{bud32} (E292R), \textit{tlc1} \textit{bud32} (E295K) mutants (in E), \textit{tlc1} \textit{bud32}(K52A) and \textit{tlc1} \textit{bud32}(N166A) mutants (in F).

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Figure 7. General homologous recombination activities in nam7Δ, ebs1Δ, upf3Δ, nmd2Δ, rps16bΔ, soh1Δ, and cgi121Δ mutants, and break-induced-replication efficiencies in ies1Δ, ies2Δ, pif1Δ, rad6Δ, and cgi121Δ mutants. (A) Inter-chromosomal recombination assay in the indicated homozygous diploid deletion mutants. (B) Intra-chromosomal recombination assay in the indicated haploid mutants. (C) Intra-chromosomal recombination assay in the indicated homozygous diploid deletion mutants. The upper panels are the schematic illustrations of each recombination event. The lower panels show the measured recombination rates in the indicated mutants. These assays were performed as reported previously [72,73] and the statistical significance was indicated as follows: **P-value<0.02 and *P-value<0.05. The rad50Δ mutant was included as a
Identification of Telomere Recombination Regulator

positive control. We failed in constructing soh1Δ homozygous mutants in (A) and (C) because the mating efficiency of this mutant was extremely low. (D) Schematic illustration of the system to detect break-induced-replication (BIR) efficiencies as reported in Lydeard et al [74]. After galactose induction, HO endonuclease causes a break in the indicated site. BIR repair process generates an intact CAN1 marker which can be detected by PCR procedures using primers P1 and P2. (E and F) BIR efficiencies were measured in pif1Δ, ies1Δ and ies3Δ mutants (E), and rad6Δ and cgi121Δ mutants (F). Semi-quantitative PCR was used to measure BIR efficiency as shown in Figure S6.

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Rad51-dependent BIR process we used a system developed by Lydeard et al. to measure the BIR efficiencies in ies1Δ, ies3Δ, pif1Δ, rad6Δ and cgi121Δ mutants [74] (Figure 7D). The rad51Δ and rad59Δ mutant strains served as positive controls [14]. Our results showed that similar to the rad51Δ mutant, the pif1Δ mutant displayed little BIR efficiency (Figure 7E). In ies1Δ, ies3Δ and rad6Δ mutants, the BIR efficiencies were greatly decreased as also seen in the rad59Δ mutant (Figure 7E and 7F). In contrast, the BIR efficiency in the cgi121Δ mutant was comparable to that of the wild-type strain (Figure 7F). Taken together, these data indicate that Pif1 is required for Rad51-dependent break-induced-replication, and the INO80 complex and Rad6, but not the KEOPS complex, contribute to this BIR process.

Discussion

Unlike most other chromosomal loci, eukaryotic telomeres have unique structures attributed to their repetitive DNA sequence and binding proteins [75]. Linear chromosome ends can be recognized as DNA double-stranded breaks and are thus often subjected to repair by non-homologous-end-joining and homologous recombination. It is possible that telomerase-null senescing cells are able to escape the fate of death as telomeres undergo lengthening and repair via homologous recombination. The distinct DNA makeup of Type I and Type II recombinational telomeres allowed us to carry out a genetic screening to identify genes that affect telomere recombination in telomerase-null cells.

Our candidate approach for screening telomere recombination genes had a few shortcomings. In our screening we only covered the 280 known TLM genes, which make up only 5.6% of the ~5,000 non-essential genes in S. cerevisiae. It would be ideal to cover all non-essential genes in our screen. However, such a study would be too massive to undertake since the screening procedures included knocking out TLC1 in every strain, two to three-weeks passaging cells until they reach senescence and Southern blot experiments for multiple survivors in each mutant (see Figure 1A and Figure 4A). The candidate approach we chose therefore had a strong bias. As a result, we might have missed potential genes that do not affect telomere length, but play important roles in telomere recombination. Another challenge to our screening approach came from the nature of different growth rates of the various mutants. Although we used heterozygous diploid mutants to generate spores of tle1Δ tlmΔ double mutants (Table S1), for quite a few mutants we were not able to distinguish between a defect in a survivor pathway and synthetic lethality (Table S1). The third issue that we were not able to resolve was to distinguish between hypo-Type I recombination and hyper-Type II recombination. The decrease of Type I survivor frequency seen in the mutants, such as spa14Δ tle1Δ (Figure 1C) could be caused by either inhibition of Type I recombination or promotion of Type II recombination. In some Type II survivors, the amplified Y'-elements were detected in Southern blot assays (Figure 1C, Figure S1), suggesting that the increase of Type II survivor frequency in these mutants was a result of enhanced Type II recombination rather than inhibited Type I recombination. This model is supported by the observation that in the nine mutants shown in Figure 1C and Figure S1, the emerging events of Type I survivors were significantly reduced, but were not entirely blocked. The fourth issue that we had not taken into consideration during our primary screening was the effect of the initial telomere length of each mutant on the recombination pathways. It was recently proposed that longer telomeres, like those observed in rj1Δ and rj2Δ mutants could influence the type of recombination pathway used at the telomere [37]. Additionally, it was shown that the mre11-A470T tlc1Δ mutant promotes telomere recombination and bypass senescence efficiently because the Type I recombination occurs before growth limitation [76]. Therefore, it would have been more appropriate to perform all of our screening steps starting with TLC1/tlc1Δ TLM/tlmΔ diploids to obtain tle1Δ single and tle1Δ tlmΔ double mutants following tetrad dissection. The fifth issue with our screening approach was that we assumed the TLM genes only affect Type I or Type II recombination. Surprisingly, the telomere structure in the yku and pif1 mutants might actually be different from that of a typical Type I or Type II survivor (Figure 3 and Figure 4). Therefore, genes that influence pathway(s) of telomere recombination other than that of Type I or Type II might have been overlooked. The sixth issue with our screen was that we only identified ten novel genes affecting Type I survivor formation (Table 1). This number might be underestimated the true total because our primary screening was carried out with a relatively stringent criteria and as such we may have overlooked some genes that have minor influences on the frequency Type I survivor emergence.

Although our screening approach had some imperfections, we successfully identified thirty-two TLM genes that influence telomere recombination when overcoming senescence. Ten of these TLM genes affected the emerging frequency of Type I survivors while twenty-two were required for Type II survivor generation. A large portion of 280 TLM genes have not previously been characterized for their roles in telomere function other than the length of the telomeres in these deletion strains was altered. The positive results of our screen provide more direct evidence supporting the idea that some of these uncharacterized TLM genes do affect telomeres [23,24,26]. Additionally, telomere recombination is a means by which cells repair defective telomeres and thus the genes involved in telomeric DNA recombination may also play a role in general DNA recombination/repair. Indeed, the TLM genes that affected either Type I or Type II recombination were also required for general DNA recombination (Figure 7A–7C). The annotated functions of the thirty-two genes that we identified point to several pathways that might contribute to telomere maintenance (Table 1 and Table 2). Some of the genes are known for functions like “rRNA processing,” “structural constituent of ribosome,” and “transport and membrane.” These gene products seem unlikely to play a direct role in telomere recombination. In contrast, the Pif1 helicase and the KEOPS complex are involved in “telomere capping and maintenance” [29,35] and INO80 complex and Rad6 are associated with “chromatin remodeling and modification.” These genes are likely to play direct roles in telomere recombination.

The seneing pif1Δ tle1Δ cells did not produce Type I survivors on solid medium (Figure 3C) and the rad50Δ pif1Δ tle1Δ triple mutant was not able to generate survivors in liquid medium (Figure 3E). These results indicated that Pif1 was required for Type I survivor generation. Interestingly, not all the rad51Δ pif1Δ
The KEOPS complex or its subunit(s) are involved in several kinases activity while Kae1 maintains endopeptidase activity [29]. The KEOPS complex facilitates the formation of the telomeric 3‘-overhang and promotes recombination of TG-tracts. Coincidently, SUA5, a telomeric single-stranded DNA binding protein, is required for both Type II recombination and t6A modification of tRNA [18,79]. It will be interesting to determine whether SUA5 is a downstream target of the KEOPS complex and if it functions in the same pathway in regulating telomere recombination. It is possible that Sua5 is a substrate of the Bud32 kinase.

In summary, our screen identified dozens of genes that regulate telomere recombination pathways. Because of the complexity of the recombination process, the molecular mechanisms of telomere recombination remain elusive. Our work not only provides important clues for beginning to understand how telomere recombination is coordinated, but also offers new insights into general DNA repair processes via homologous recombination.

Materials and Methods

Yeast strains and plasmids

All strains used in this work are summarized in Table S1 and Table S3. Gene deletions were carried out using standard procedures by genetic cross and homologous recombination. Systematic deletion strains are from EUROSCARF. We constructed CEN plasmids pRS316-PIF1, pRS313-KAE1 and pRS313-BUD32 by inserting fragments (from upstream 1000 bp to downstream 500 bp of genes’ open reading frame) into the pRS316 or pRS313 vector. Point mutations were introduced using a site-directed mutagenesis method.

Cell viability assay

A single colony of the indicated yeast strains was streaked on YPD plate and grown until emergency of single colonies (25 cell density) on YPD medium. This procedure was repeated for up to 14 times, unless the cell density is too low for dilution.

Single-colony streaking assay

A single colony of the indicated yeast strains was streaked on YPD plate and grown until emergency of single colonies (25 cell divisions) at 30°C. Individual colonies were restreaked repeatedly at least six times to allow survivors to generate.

Telomere Southern blot

Genomic DNA was prepared from each strain, digested with XhoI, separated on 1% gel, transferred to Hybond-N+ membrane (GE Healthcare) and then probed with TG1–3 telomere-specific probe or Y-element probe [23]. The CDC15 probe was ~263 bp sequence of CDC15 gene [50].

General recombination assays

Recombination assays for intrachromosomal and interchromosomal recombination in haploid and diploid strains were performed and recombination rates were determined as previously described [72,73]. For each mutant, about 2×10^7 yeast cells were plated on solid selective medium. After growing at 30°C for 2–3 days, about 200 positive colonies would appear on the plate in wild-type haploid strain. Recombination rates were calculated and statistically analyzed by paired two-sample t-test.

Measurement of break-induced-replication efficiency

Break-induced-replication (BIR) efficiency was measured in a system developed by Lydeard et al [74]. Semi-quantitative PCR
was conducted as previously described [74]. PCR products were quantified in Image Quant Software.

**Supporting Information**

**Figure S1** Southern blot analysis of survivor types in tlc1Δ tlmΔ double mutants. The tlmΔ tlc1Δ double mutants were generated through tetrad dissection from heterozygous diploids with one copy of TLM gene and TLC1 deleted. The mutants tested and shown are (A) rjiαA tlc1Δ, (B) rjiαA tlc1Δ, (C) sap3Δ tlc1Δ, (D) rpb6Δ tlc1Δ, (E) soh1Δ tlc1Δ, (F) rpb6Δ tlc1Δ, (G) rps16Δ tlc1Δ and (H) gyp1Δ tlc1Δ. Fifty independent colonies of each mutant were randomly selected and passed on solid plates, and the telomere structures of survivors were examined by Southern blot using a TG probe. The triangles (▼) indicate Type I survivors, while the others are Type II survivors. The frequencies of Type II survivors were calculated and summarized in Figure 2B (column of “Spores from tetrad dissection”). (TIF)

**Figure S4** The effect of thirteen DNA helicase genes on survivor formation. Thirteen DNA helicase genes were knocked out in a TLC1 deletion mutant. These double mutants were either passaged on plates (A) or serially cultured in liquid medium (B) until survivors generated. The telomere structures of survivors were examined by Southern blot assay. (A) On plates, only the pif1Δ tlc1Δ mutant could not form Type I survivors. (B) In liquid cultures, the sgs1Δ tlc1Δ mutant could only form Type I survivors. (TIF)

**Figure S5** Cell viability assay of rad6Δ and its downstream target gene mutants. The heterozygous diploid TLC1/tlc1Δ RAD18/rad18Δ BREL1/brel1Δ UBR1/ubr1Δ mutant was sporulated and tetrads were dissected. One spore of tlc1Δ single mutant and three spores of each genotype of (A) ubr1Δ tlc1Δ, (B) rad18Δ tlc1Δ, (C) brel1Δ ubr1Δ tlc1Δ, (D) brel1Δ rad18Δ tlc1Δ, (E) ubr1Δ rad18Δ tlc1Δ and (F) brel1Δ ubr1Δ rad18Δ tlc1Δ were subjected to cell viability assay and the corresponding strains are indicated in each panel. (TIF)

**Figure S6** Representative gels of the BIR repair product in wild type, rad33Δ, rad39Δ, ies1Δ, ies3Δ, pif1Δ, rad6Δ and sgs12Δ cells. Cells were harvested at 0, 1, 5, 6, 8, 10, 12 and 24 hr after HO induction, then the genomic DNA was extracted and subjected to semi-quantitative PCR. The BIR repair products were labeled as “CANT” and reference PCR products of the FLJ9 locus were displayed as loading controls. (TIF)

**Table S1** Complete list of 290 TLM genes. (XLS)

**Table S2** List of screened-out genes which had been reported in DNA repair and recombination. (DOC)

**Table S3** Yeast strains used in this study. (DOC)

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**Author Contributions**

Manuscript preparation: H-BT N-NL JP. Conceived and designed the experiments: YH F-LM J-QZ. Performed the experiments: YH H-BT N-NL JP. Analyzed the data: YH H-BT N-NL J-QZ JP. Wrote the paper: YH J-QZ.
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