SUPPLEMENTAL INFORMATION

Data S1, related to Figures 5 and 6

Detailed description of ALT survivor chromosome end structures as determined by Oxford Nanopore Technology (ONT) sequencing.

Structural analysis of the parental strain AM3692

The analysis of telomere length and Y' regions were performed by a combination of ONT and PacBio sequencing. The length of individual telomeres was determined by ONT sequencing of the longest three reads, representing each telomere, and demonstrated that the median telomere length was 278 (average 275) with a range of 163-338bp (See Table S3 for the telomere length for each telomere). The number of Y' regions was determined based on ONT sequencing of the longest read, (which was sufficient since ONT errors did not compromise recognition of Y' regions) and demonstrated that AM3692 contained 11 Y' regions (4 Long Y', and 7 Short Y' Figures 5A, 5B and Table S3). This conclusion was confirmed by the results of PacBio sequencing, as well as by using ddPCR normalized to the *ACT1* gene. To remove ONT sequencing errors, a consensus sequence for each Y' was built by aligning six reads using CLCgenomics12 (see STAR Methods for parameters). Using these consensus sequences, phylogenetic analysis was performed by nucleotide distance-based clustering using MEGAX (Kumar et al., 2018), (see STAR Methods for details), which demonstrated that six Y' regions in AM3692 were significantly different from all Y' in the strain (Figures 5A and 5B), while the remaining five were separated into two groups with no significant difference of Y' inside each group (Figures 5A and 5B). Of the two separate groups, Group 1 contains ChrX-L and XVI-R and Group 2 contains the Y' from ChrVI-L, ChrVIII-R, and XIV-R. Together, we can distinguish and separate 8 types of Y' regions (6 unique and 2 separate groups) (Figures 5A and 5B).

Chromosome structure of ALT survivor ZK-1 obtained in *tlc1Δ* strain.

ZK-1 is an ALT survivor (Figures 5D; Table S3) formed after passaging *tlc1::BSD* cells in liquid cultures (similar to Figure 1B). Based on ONT sequencing, the median telomere length in this survivor was 588bp and average of 565bp (range of 103-1103bp), which is within published Type II telomere lengths (Chen et al., 2001; Le et al., 1999; Teng et al., 2000) and is significantly longer (P-value <0.0001,determined by Mann-Whitney test) as compared to the parental **AM3692** (Figure 5A; Table S3). Based on ONT results (and using the longest ONT read) we determined that this survivor contains 11 pre-telomeric Y' sequences, which was confirmed by ddPCR. Importantly, even though **ZK-1** contains the same number of Y' as

AM3692 only 6 out of 11 original Y' regions have been maintained (ChrV-L, V-R, VI-L, VI-R, VIII-R XIV-R) (based on the results of MEGAX analysis, see STAR Methods for details). Further, four Y' on chromosomes ChrI-R, IX-R, X-L, XI-R were replaced with Y' Group 1 or 2 sequences. Finally, one chromosome end (ChrXVI-R) lost Y', and one other end (ChrIII-L) gained a Y' from Group 1. Together, this survivor is similar to the Type II in structure since it contained elongated telomeres and did not contain tandem Y' sequences. However, **ZK-1** had many changes in Y' indicative of active recombination at Y's.

Chromosome structure of ALT survivor ZK-3 obtained in *tlc1Δ rad51Δ* strain.

ZK-3 is an ALT survivor (Figure 5E; Table S3) formed after liquid passaging (similar to Figure 1B) of *tlc1::BSD rad51::KAN* cells (derivative of AM3858). Based on ONT sequencing, telomere length was determined to have a median of 924bp and average of 908bp (range of 203-1654bp), which is within known Type II telomere lengths and are significantly longer (P-value <0.0001) as compared to the parental **AM3692** (Figure 5A; Table S3). Based on ONT results, using the longest read, we determined that this survivor contains 14 pre-telomeric Y' sequences, which is confirmed by ddPCR. Phylogenetic analysis demonstrated that 5 Y' were maintained in the same way as they were in the parental strain, which included (ChrV-L, VI-L, VI-R, VIII-R, XIV-R) Y' sequences. Further, we observed that 4 Y' regions (IX-R, X-L, XI-R, XVI-R) were replaced by Y' from group 1 or 2 Y' sequence, while two others (ChrI-R and ChrVR) were also replaced, but the exact source remains unclear. In addition, 3 chromosomal ends (ChrIII-R, XII-R, XIII-R) that did not originally have a Y' acquired a Y' from ChrI-R or Group 2 sequences. Thus, recombination involving Y's, which was previously believed to be Rad51-dependent, can occur even in the absence of Rad51. Otherwise, **ZK-3** was similar to "classic" Type II type survivors since it contained stable long telomeres and had no Y' tandems.

Chromosome structure of ALT survivor ZK-23 obtained in *tlc1Δ rad51Δ* strain.

ZK-23 (Figure S5B; Table S3) is an ALT survivor formed after plating (similar to Figure 1C) of *tlc1::BSD rad51::KAN* cells (derivative of AM3858). Based on ONT sequencing, telomere length was determined to have a median of 877bp (average 862bp; range of 183-1988bp), which is within known Type II telomere lengths and telomeres in this survivor were significantly longer (P-value <0.0001) as compared to the parental **AM3692** (Figure 5A;Table S3). Based on ONT results, we determined that this survivor contained 8 Y' sequences, which was confirmed by ddPCR. Phylogenetic analysis demonstrated that there were 4 Y' regions in this survivor that were the same as in the parent (ChrV-R, VI-R, VIII-R, XI-R). In addition, 3 Y' regions (ChrIX-R, X-L, XVI-R) were replaced with another Y' region from ChrXI-R, or Group 1. Also, 3

chromosomal ends (ChrI-R, V-L, VI-L) lost their Y' and one (ChrXI-L) gained a new Y' region from group 1 (Table S3). Together, this survivor was similar to Type II in structure since it contained elongated telomeres and did not contain tandem of Y' sequences.

Chromosome structure of ALT survivor ZK-4 obtained in *tlc1Δ rad59Δ* strain.

ZK-4 (Figure 5F; Table S3) is an ALT survivor formed after passaging of *tlc1::BSD rad59::KAN* cells (derivative of AM4950) according to schematic in Figure 1B. Based on ONT sequencing, telomere lengths for individual chromosome ends were determined, and the median length was 816bp, average 841bp (range of 120-1893bp), which is within known Type II telomere lengths and significantly longer (P-value <0.0001) as compared to the parental **AM3692** (Figure 5A; Table S3). Based on ONT results we determined that this survivor contained 19 Y' sequences, which was also confirmed by ddPCR. Phylogenetic analysis demonstrated that four chromosomal ends maintained their original Y' sequence (ChrV-L, VI-L, VIII-R, XIV-R). Also, at seven chromosome ends the original Y's were replaced by other ones, in particular ChrI-R, V-R, VI-R, IX-R, X-L, XI-R, XVI-R were replaced by group 1 or 2 Y' sequences. Finally, 8 chromosome ends that originally did not contain Y's (ChrII-L, II-R, IV-L, VII-L, IX-L, XIII-R, XIV-L, XVI-L) acquired them. Specifically, five of them acquired Y' belonging to Group 2 and three acquired Y' sequences from Group 1 Y' (Figure 5F; Table S3). Together, this survivor formed in *tlc1Δ rad59Δ* contained long telomeres, but no Y' tandems, indicative of a Type II survivor, which is interesting since previous reports suggested that *tlc1Δ rad59Δ* can produce only Type I survivors.

Chromosome structure of ALT survivor ZK-17 obtained in *tlc1Δ* strain.

ZK-17 is an ALT survivor (Figure 6A; Table S3) formed after passaging *tlc1::BSD* cells in liquid cultures similarly to (Figure 1B). Based on ONT sequencing, we determined that this survivor contained 152 pre-telomeric Y' sequences, while ddPCR indicated the presence of 556 Y' sequences (Figure 6A; Table S3). Based on this, we hypothesized that this survivor had tandems of Y' formed on its chromosomal ends, and these tandem Y' were so long that ONT sequencing was unable to cover the entirety of the sequence between the bait sequence and the telomere. Taking in account that we have already obtained >7 Gb of sequence reads for this survivor and they were sufficiently long, many upwards of 100kb, it was likely that the tandems of ends that we were not able to sequence were longer than 100kb making it difficult to reach all telomere ends with further sequencing. Based on analysis of individual ONT reads, we found that out of 32 chromosome ends, 25 contained Y' tandems, while 7 did not (Figure 6A; Table S3). Among those that contain Y' tandems, only 8 reached the telomere end while the remaining

17 stopped within a Y'. Using the chromosome ends where the entire Y' tandems were sequenced through the telomere; we estimated the average number of Y' in tandems as 7.1 with a range of 2-16 (Figure 6A; Table S3). However, this is certainly an underestimation since we likely reached the ends of the shortest Y' tandems. Next, we created consensus sequences for individual Y' that were covered by at least 6 reads and created phylogenetic trees based on these consensus sequences. We then looked at each individual Y' tandem and found that 21 out of 23 tandems (where origin could be identified in multiple Y'), consisted of Y' that were indistinguishable from each other within each tandem (Figure 6A). The remaining 2 maintained their original Y', but then added Y' tandems from a single source. The observation that individual Y' are iterated within the tandems allowed us to propose that the tandem Y' could be formed by multiple re-invasions or by rolling circle replication. It was determined that the median telomere length in this survivor (**ZK-17**) was 488bp, average 755 (with a range of 79-1985bp), which is within known Type II telomere lengths, significantly longer ($P < 0.0001$) as compared to the parental **AM3692** (Figure 5A; Table S3). Together, this survivor contained tandem Y' sequences along with long telomeres which makes us to classify this survivor as a "hybrid" Type I/II survivor.

Chromosome structure of ALT survivor ZK-2 obtained in *tlc1Δ* strain.

ZK-2 is an ALT survivor (Figure 6B; Table S3) formed after plating of *tlc1::BSD* cells (similar to Figure 1C). Based on ONT sequencing, we determined that this survivor contained 182 Y' pre-telomeric sequences (Figure 6B; Table S3). According to ddPCR, the number of Y' sequences in this survivor was 198, which is close 182, and the small difference may result from heterogeneity of the sample, see below. These Y's formed tandems on each chromosomal end with an average of 5.9 Y' per chromosome end (range of 2-13) (Figure 6B; Table S3). Attempts of creating the consensus sequence for individual Y' in this survivor proved that the culture contained heterogeneous chromosome ends. In particular, it was impossible to align the six individual reads representing the Y' located in the same position of the tandem. We propose that the telomeres in this survivor were not stabilized and remained short, and the culture continued to recombine. Indeed, the length of the telomeres in this survivor, determined by the longest reads, had a median of 140 bp (average of 141bp; range of 45-222), which means that telomeres were significantly shorter as compared to the parental **AM3692** strain ($P < 0.0001$) (Figure 6B; Table S3). The idea that this resulted from a dynamic telomere process was further supported by measuring the number of Y' after further passages of ZK-2 in liquid culture by using ddPCR. Specifically, the number of Y' that was 198 at the beginning was progressively

changed to 308, 81, 55, 139, 248, 354, 65, 355, 132, and 123 in subsequent passages 1 through 10 (Figure 6C). Overall, we propose that what is usually considered to be a Type I survivor (Le et al., 1999; Lundblad and Blackburn, 1993; Teng and Zakian, 1999) represents an unstable cells population undergoing a highly dynamic process of telomere maintenance.

Chromosome structure of ALT survivor ZK-18 obtained in *tlc1Δ* strain.

ZK-18 is an ALT survivor (Figure 6D; Table S3) formed after 10 passages of **ZK-2** at 250 cells/ml, where the total number of Y' fluctuated at each passage (Figure 6D). Based on ONT sequencing, we determined that this survivor contained 194 pre-telomeric Y' sequences. Based on analysis of individual ONT reads, we found that of 32 chromosome ends, 30 contained Y' tandems, while 2 did not (single Y') (Figure 6D; Table S3). Among those that contain Y' tandems, 22 reached the telomere ends while the remaining 10 stopped within a Y'. It was determined that the median telomere length in this survivor was 984.5bp (average of 985bp; range of 161-2121bp), which is within known Type II telomere lengths, significantly longer ($P < 0.0001$) as compared to the parental **AM3692** (Figure 5A; Table S3). Therefore, the additional passages allowed the unstable **ZK-2** to gain long telomeres and to become a "hybrid" type ALT outcome. Together this supports that the classic Type I ALT outcome **ZK-2** is an intermediate of the ALT formation pathway that can be stabilized by long telomeres with further passaging to become a "hybrid" ALT outcome.

Chromosome structure of ALT survivor ZK-5 obtained in *tlc1Δ* strain.

ZK-5 is an ALT survivor (Figure S5A; Table S3) formed after plating (similar to Figure1C) of *tlc1::BSD* cell. Based on ONT sequencing, we determined that this survivor contained 131 Y' pre-telomeric sequences. According to ddPCR, the number of Y' sequences was 67, such a difference may result from heterogeneity of the sample. Attempts at creating the consensus sequence for individual Y' proved that the culture contained heterogeneous chromosome ends. We propose that, similar to **ZK-2**, the telomeres in this survivor were not stabilized and remained short, and the culture continued to change the chromosome ends. Indeed, the median length of the telomeres in this survivor determined by the longest reads was 115bp (average of 109bp; range of 20-171bp) (Figure S5A; Table S3), which means that telomeres were significantly shorter as compared to the parental **AM3692 strain** ($P < 0.0001$). Together **ZK-5** was a classic Type I that maintains telomeres though a dynamic process, similar to **ZK-2** (Figure 6B), which also formed a heterogeneous population.

Chromosome structure of ALT survivor ZK-33 obtained in *tlc1Δ rad59Δ* strain.

ZK-33 is an ALT survivor formed after plating (similar to Figure 1C) of *tlc1::BSD rad59::KAN* cells (Figure S5C; Table S3). Based on ONT sequencing, we determined that this survivor contained 252 Y' pre-telomeric sequences. According to ddPCR, the number of Y' sequences was 198, and the difference between these two measurements may result from the heterogeneity of the sample. These Y's formed tandems on each chromosomal end with an average of 8 Y' per chromosome end (range of 2-19). Attempts at creating the consensus sequence for individual Y' proved that the culture contained heterogeneous chromosome ends. Based on ONT sequencing the telomere length was determined to have a median of 83bp (average of 90bp; range of 52-153bp) (Figure S5C; Table S3), which was within known Type I telomere lengths significantly shorter ($P < 0.0001$) as compared to the parental **AM3692.** Together **ZK-33** was a classic Type I that maintained telomeres though a dynamic process, similar to **ZK-2** (Figure 6B) and **ZK-5** (Figure S5A) and formed a heterogeneous population of cells.

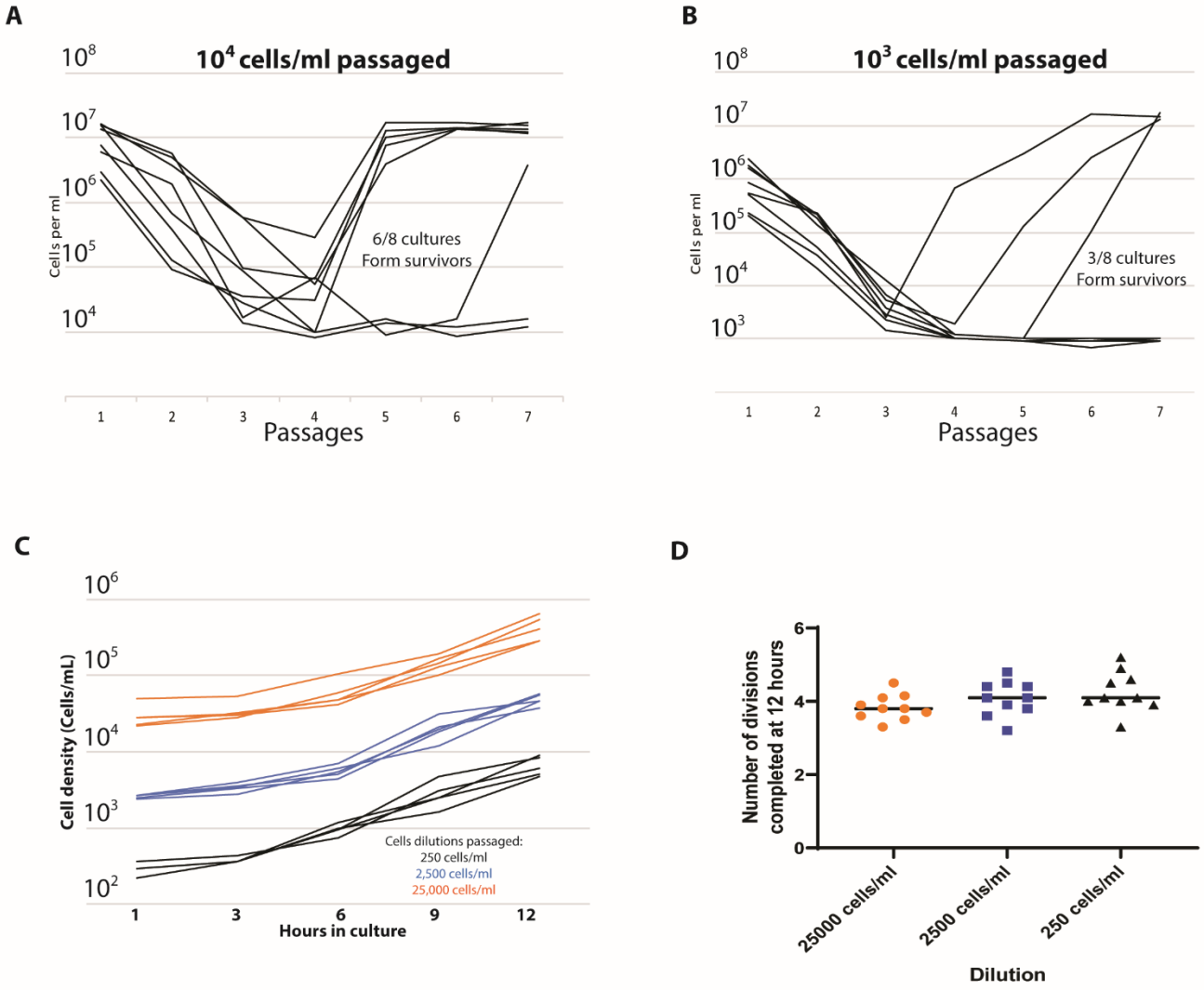

A
10^4 cells/ml passaged
Cells per ml
6/8 cultures
Form survivors
Passages
B
10^3 cells/ml passaged
Cells per ml
3/8 cultures
Form survivors
Passages
C
Cell density (Cells/mL)
Cells dilutions passaged:
250 cells/ml
2,500 cells/ml
25,000 cells/ml
Hours in culture
D
Number of divisions completed at 12 hours
25000 cells/ml
2500 cells/ml
250 cells/ml
Dilution

Figure S1. Determining the parameters for frequency analysis of ALT survivors, Related to Figure 1.

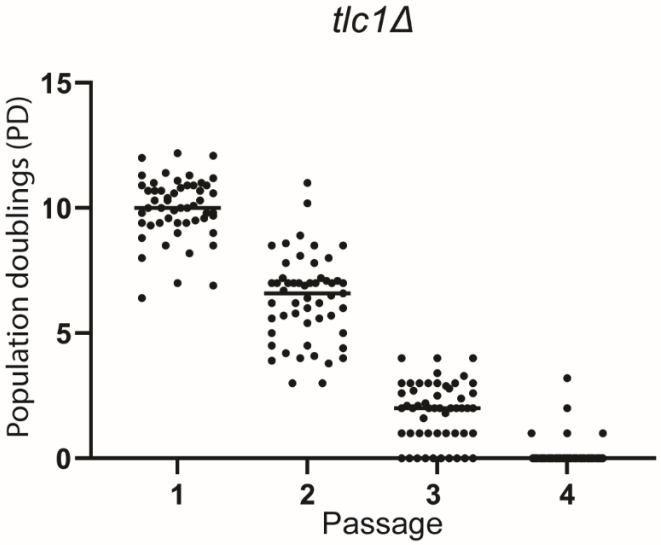
(A-B) The occurrence of ALT survivors decreases with a reduction of passaged population size (10^4 cells/ml in (A) versus 10^3 cells/ml in (B)). The proportions indicate the number of experiments where survivors formed out of a total of 8 experiments.

(C) The dynamics of *tlc1Δ* culture growth rate with varying initial cell concentrations ($2.5x10^4$, $2.5x10^3$, and $2.5x10^2$ cells/ml).

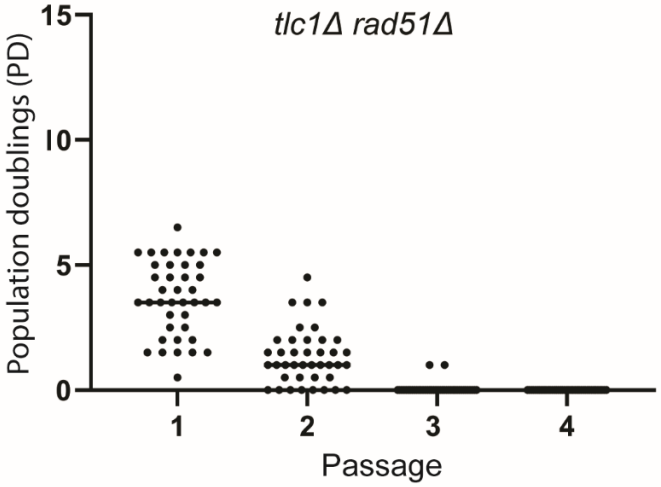
(D) Total number of PD completed by *tlc1Δ* cultures at the end of 12 hours are the same for three different initial cell concentrations ($2.5x10^4$, $2.5x10^3$, and $2.5x10^2$ cells/ml).

(See also Tables S1 and S2)

A


tlc1Δ
Population doublings (PD)
15
10
5
0
1
2
3
4
Passage

B


tlc1Δ rad51Δ
Population doublings (PD)
15
10
5
0
1
2
3
4
Passage

C

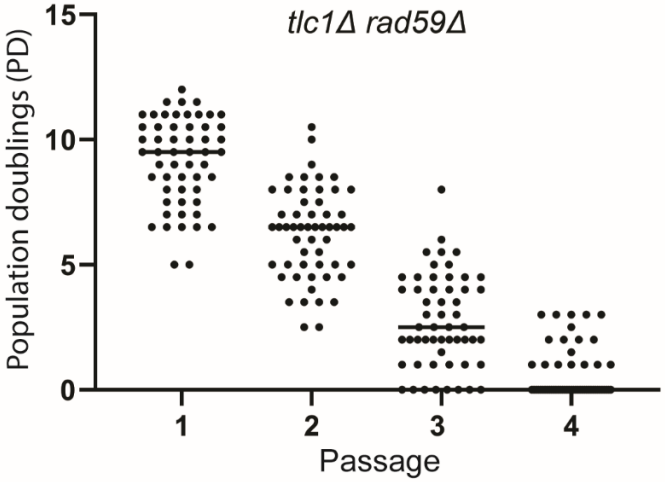

tlc1Δ rad59Δ
Population doublings (PD)
15
10
5
0
1
2
3
4
Passage

Figure S2. The number of population doublings following the first four passages in each liquid culture, Related to Figures 2 and 3.

(A) The individual points represent the number of doublings that occur in each specific *tlc1Δ* passage of each culture, with lines representing the median values.

(B) Same as (A), but for *tlc1Δ rad51Δ*

(C) Same as (A), but for *tlc1Δ rad59Δ*.

(See also Tables S1 and S2)

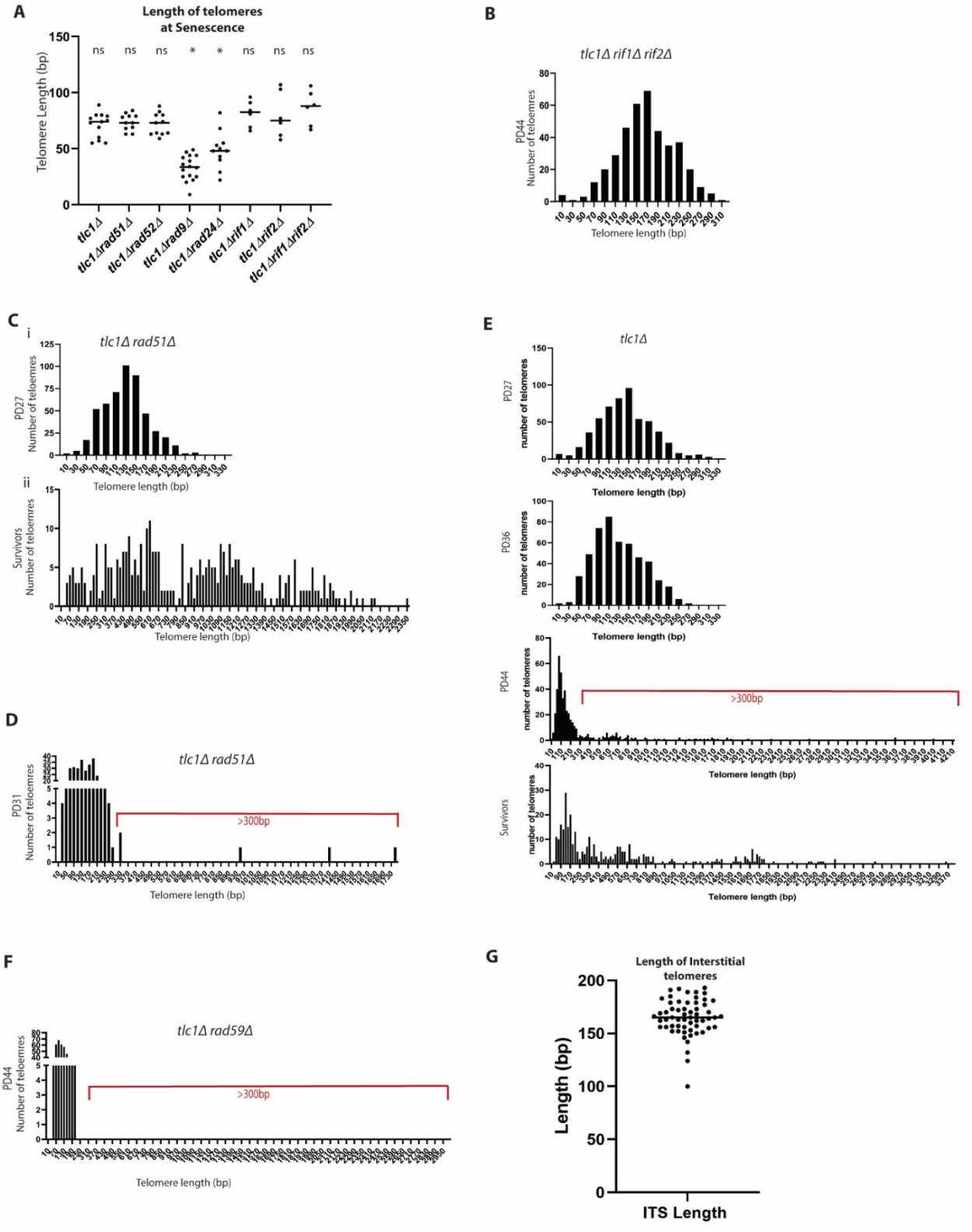

A
Length of telomeres at Senescence
Telomere Length (bp)
ns ns ns * * ns ns ns
tlc1Δ
tlc1Δrad51Δ
tlc1Δrad52Δ
tlc1Δrad9Δ
tlc1Δrad24Δ
tlc1Δrif1Δ
tlc1Δrif2Δ
tlc1Δrif1Δrif2Δ
B
tlc1Δ rif1Δ rif2Δ
PD44
Number of teloemres
Telomere length (bp)
C
i
tlc1Δ rad51Δ
PD27
Number of teloemres
Telomere length (bp)
ii
Survivors
Number of teloemres
Telomere length (bp)
D
tlc1Δ rad51Δ
PD31
Number of teloemres
>300bp
Telomere length (bp)
E
tlc1Δ
PD27
number of telomeres
Telomere length (bp)
PD36
number of telomeres
Telomere length (bp)
PD44
number of telomeres
>300bp
Telomere length (bp)
Survivors
number of telomeres
Telomere length (bp)
F
tlc1Δ rad59Δ
PD44
Number of teloemres
>300bp
Telomere length (bp)
G
Length of Interstitial telomeres
Length (bp)
ITS Length

Figure S3. Telomere end distribution determined by Southern blot and PacBio sequencing, Related to Figures 2 and 4.

(A) Quantification of the telomere lengths 10th percentile determined via Southern blot hybridization with a Y' specific probe, for at least 6 independent experiments. Line indicates median value.

(B) Analysis of telomere lengths by PacBio sequencing in *tlc1Δ rif1Δ rif2Δ* cells grown to PD44, the lengths are in 20bp bins indicated by the middle value of the bin. The lengths are in 20bp bins indicated by the middle value of the bin.

(C) Analysis of telomere lengths by PacBio sequencing in *tlc1Δ rad51Δ* cells grown to PD27 and survivor culture. The lengths are in 20bp bins indicated by the middle value of the bin. The lengths are in 20bp bins indicated by the middle value of the bin.

(D) Telomere lengths by PacBio sequencing in an independent culture of *tlc1Δ rad51Δ* cells grown to PD31. Red bracket indicates telomere lengths compatible with ALT survivors. The lengths are in 20bp bins indicated by the middle value of the bin. The lengths are in 20bp bins indicated by the middle value of the bin.

(E) Telomere end distribution determined by PacBio sequencing (similar to Figure 4D, but for another independent culture of *tlc1Δ*). See Figure 3C for passaging scheme and legend to Figure 4D for details. Red bracket indicates telomere lengths compatible with ALT survivors. The lengths are in 20bp bins indicated by the middle value of the bin. The lengths are in 20bp bins indicated by the middle value of the bin.

(F) Analysis of telomere lengths by PacBio sequencing in *tlc1Δ rad59Δ* cells grown to PD44. Red bracket indicates telomere lengths compatible with ALT survivors. The lengths are in 20bp bins indicated by the middle value of the bin. The lengths are in 20bp bins indicated by the middle value of the bin.

(G) The length of ITS telomeres in AM3692 determined by ONT. The solid line indicates median telomere length.

Kockler et al. Figure S4

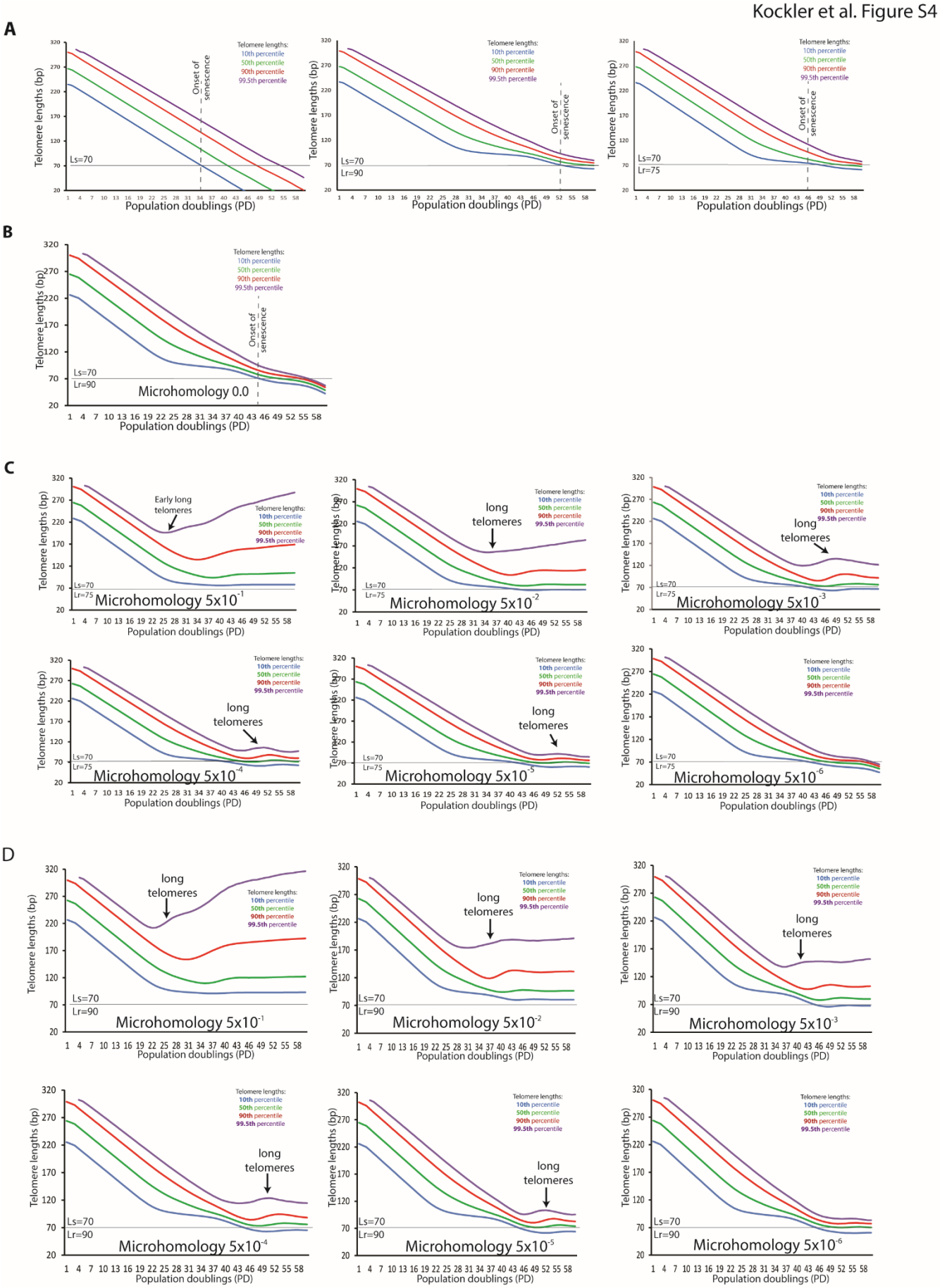


Figure S4 Computational modeling of telomere erosion and repair (at homologous and microhomology regions) with varying parameters, Related to Figures 2, 3, and 4.

(A) Dynamics of the entire distribution of telomere lengths following telomere erosion rate=5bp/div. Left: Telomere erosion alone. Middle: recombination at homology Ls=70, and Lr=90. Right: recombination at homology Ls=70, and Lr=75.

(B) Dynamics of the entire distribution of telomere lengths following telomere erosion and recombination at positions of homology, with telomere erosion rate=6bp /div., Ls=70, Lr =90.

(C) Dynamics of the entire distribution of the telomere lengths with parameters (erosion rate=6bp /div., Ls=70, and Lr=75) and with recombination at positions of homology and microhomology at various frequencies per repairing telomere (indicated).

(D) Dynamics of the entire distribution of the telomere lengths with parameters (erosion rate=6bp /div., Ls=70, and Lr=90) and with recombination at positions of homology and microhomology at various frequencies per repairing telomere (indicated).

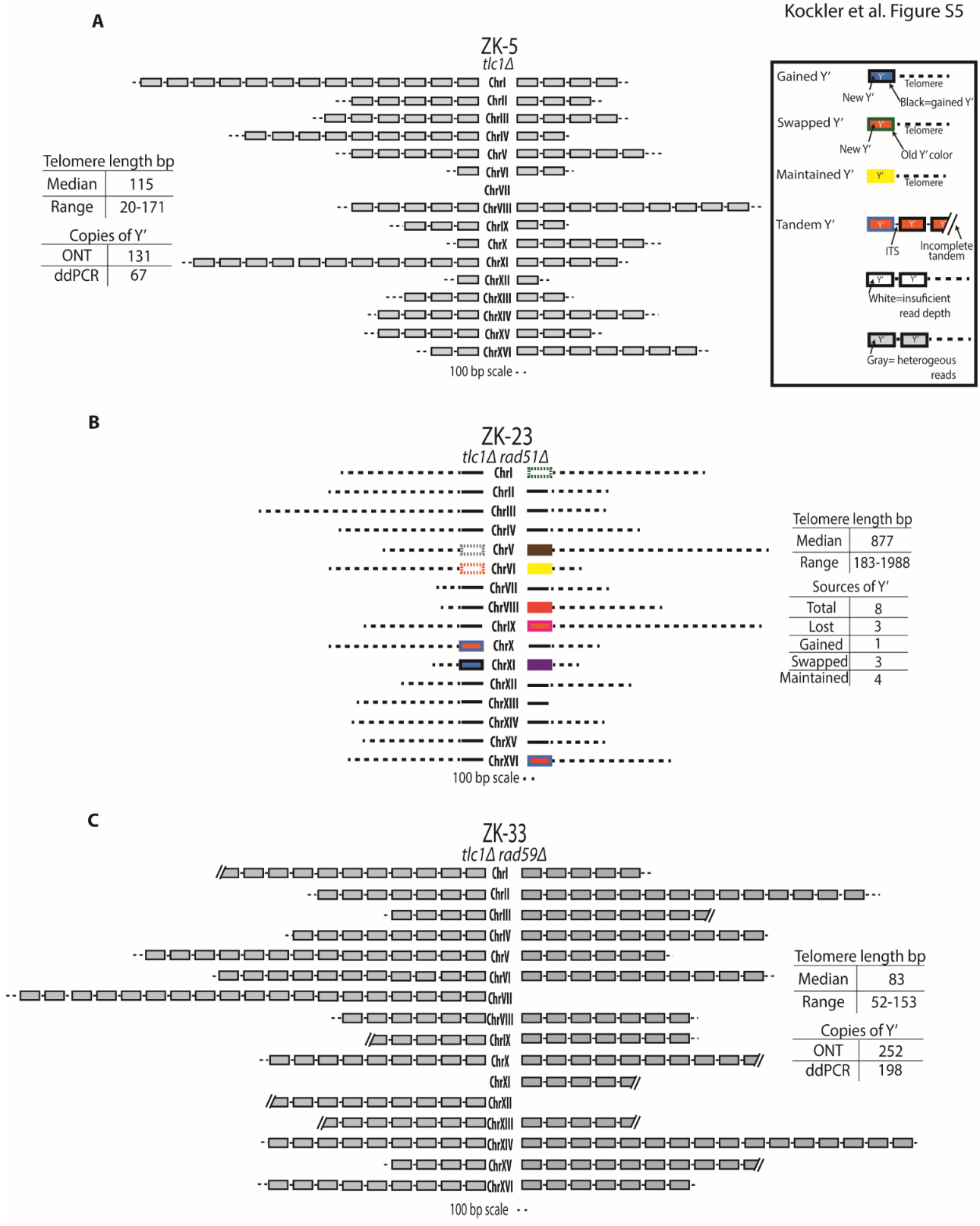

A
ZK-5
tlc1Δ
ChrI
ChrII
ChrIII
ChrIV
ChrV
ChrVI
ChrVII
ChrVIII
ChrIX
ChrX
ChrXI
ChrXII
ChrXIII
ChrXIV
ChrXV
ChrXVI
100 bp scale
Telomere length bp
Median 115
Range 20-171
Copies of Y'
ONT 131
ddPCR 67
Gained Y'
Telomere
New Y'
Black=gained Y'
Swapped Y'
Telomere
New Y'
Old Y' color
Maintained Y'
Telomere
Tandem Y'
ITS
Incomplete tandem
White=insuficient read depth
Gray= heterogeous reads
B
ZK-23
tlc1Δ rad51Δ
ChrI
ChrII
ChrIII
ChrIV
ChrV
ChrVI
ChrVII
ChrVIII
ChrIX
ChrX
ChrXI
ChrXII
ChrXIII
ChrXIV
ChrXV
ChrXVI
100 bp scale
Telomere length bp
Median 877
Range 183-1988
Sources of Y'
Total 8
Lost 3
Gained 1
Swapped 3
Maintained 4
C
ZK-33
tlc1Δ rad59Δ
ChrI
ChrII
ChrIII
ChrIV
ChrV
ChrVI
ChrVII
ChrVIII
ChrIX
ChrX
ChrXI
ChrXII
ChrXIII
ChrXIV
ChrXV
ChrXVI
100 bp scale
Telomere length bp
Median 83
Range 52-153
Copies of Y'
ONT 252
ddPCR 198

Figure S5. ONT analysis of chromosome ends of individual ALT survivors, Related to Figures 5 and 6.

(A) Unstable ALT survivor ZK-5 from *tlc1Δ,* similar to another survivor ZK-2, shown in Figure 6B, Grey fill color=Y' source cannot be determined due to heterogeneity. Lost Y'=dashed outline of original source color with no fill; Gained Y'= black outline with fill of new Y'; Swapped Y'= original Y' source outline with fill of new Y'; and Maintained Y'= outline and fill color the same color. No fill color=Y' source not determined; Gray color= heterogenous reads for Y' source.

(B) ALT survivor from *tlc1Δ rad51Δ.*

(C) Unstable ALT survivor from *tlc1Δ rad59Δ.* Grey fill color=Y' source cannot be determined due to heterogeneity.
(See also Table S3)