Supplemental Material for Cohn *et al.* , 2019

Alternative Lengthening of Telomeres in the Budding Yeast *Naumovozyma castellii*.

Supplementary Figures S1-S6

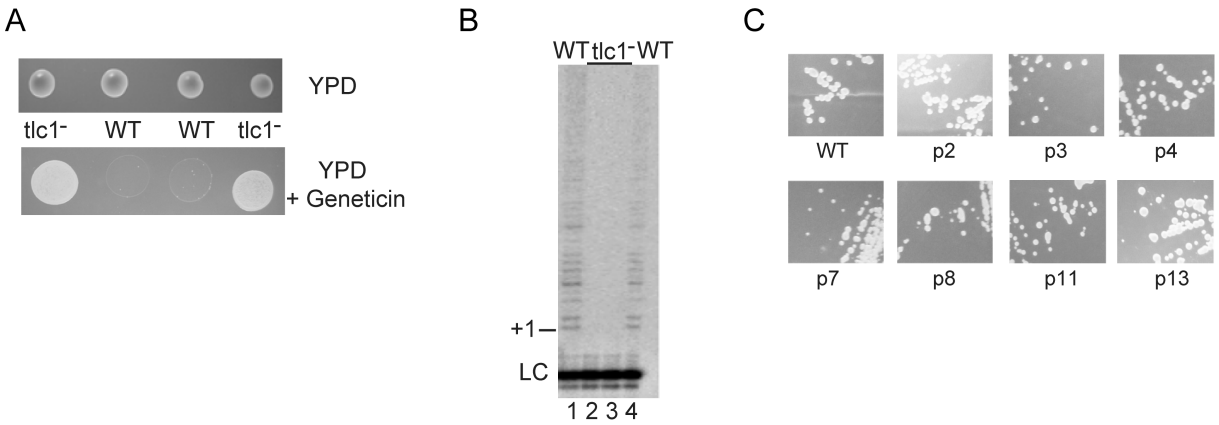


Figure S1. Tetrad analysis of telomerase knockouts. The *TLC1* gene was targeted with a *tlc1::kanMX3* cassette in the diploid yeast strain Y235. The transformants were sporulated and then microdissected on YPD germination plates. (A) Four spores from the same ascus (tetrad) grown at 25°C for 2 days (termed passage 1, p1). Lower panel shows the Geneticin sensitivity test for the identification of the knockout spores. Each spore was spotted on both YPD and YPD-geneticin plates. All spores grew on the YPD plates and only knockout spores (*tlc1::kanMX3*) grew on the YPD-geneticin plate, indicating presence of the *kanMX3* marker in the genome. (B) Telomerase assays. Primer extension assays of telomerase extracted from the WT spores (lanes 1 and 4) and *tlc1*$^-$ spores (lanes 2-3). The 16 nt primer $(GGGTGTCT)_2$ was used as substrate. LC denotes loading control and size marker (+1) indicates the first added nucleotide. (C) Close-up of diploid colonies (*tlc1*$^-$ /*tlc1*$^-$) from the plates in Figure 1B at the indicated passages (p) and wild-type (WT). The strain is *HO*$^+$, and the haploid spores become diploid after germination.

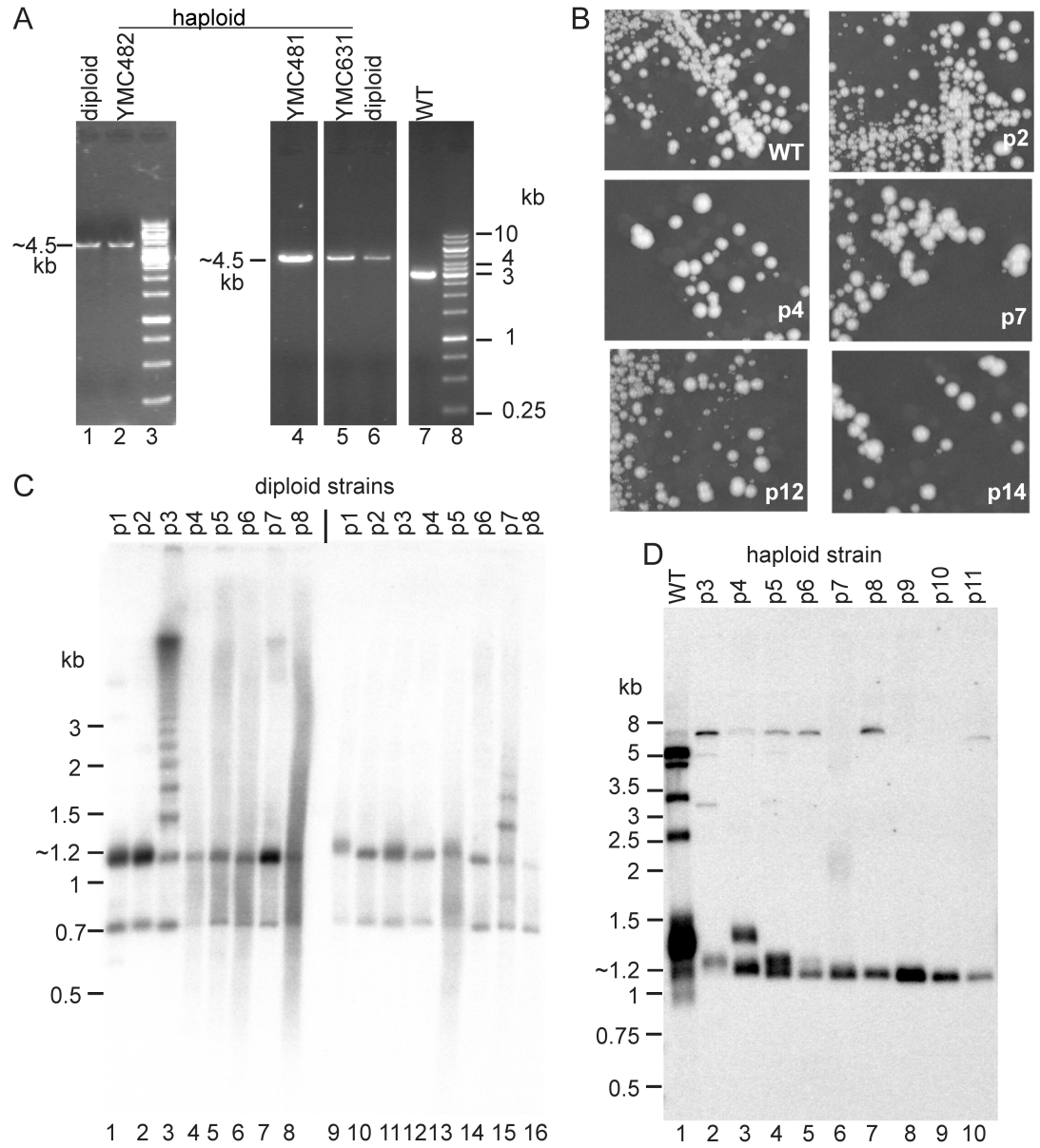


Figure S2. (A) Genotype analysis of haploid *tlc1*$^-$ strains. PCR amplicons of the *TLC1* locus were separated on an 0.8% agarose gel. The insertion of the kanMX3 marker cassette into the *TLC1* gene generates the expected 4589 bp band for the KO strains. The new haploid strains (lanes 2, 4 and 5) are compared with the diploid (*tlc1*$^-$/*tlc1*$^-$) KO strain YMC121 (lanes 1 and 6). The amplicon of WT *TLC1* generates a 3040 bp band (lane 7). DNA markers, GeneRuler 1 kb Thermo Scientific (lanes 3 and 8). (B) Close-up of haploid KO colonies (*tlc1*$^-$) and WT from the plates in Figure 2A, at the indicated passages (p). (C) TRF assays of two diploid (*tlc1*$^-$/*tlc1*$^-$) KO strains from the initial screening of spore streaks. Each strain was analyzed for 8 passages (p1-p8) including the intitial germination colony (p1). Unfortunately some degradation of the DNA occurred in some samples, resulting in low moleular weight smears. (D) TRF assay of the haploid (*tlc1*$^-$) KO strain YMC482 (p3-p11). In all TRF assays the genomic DNA was digested with *Hind*III and Southern blot hybridization performed with a telomeric probe.

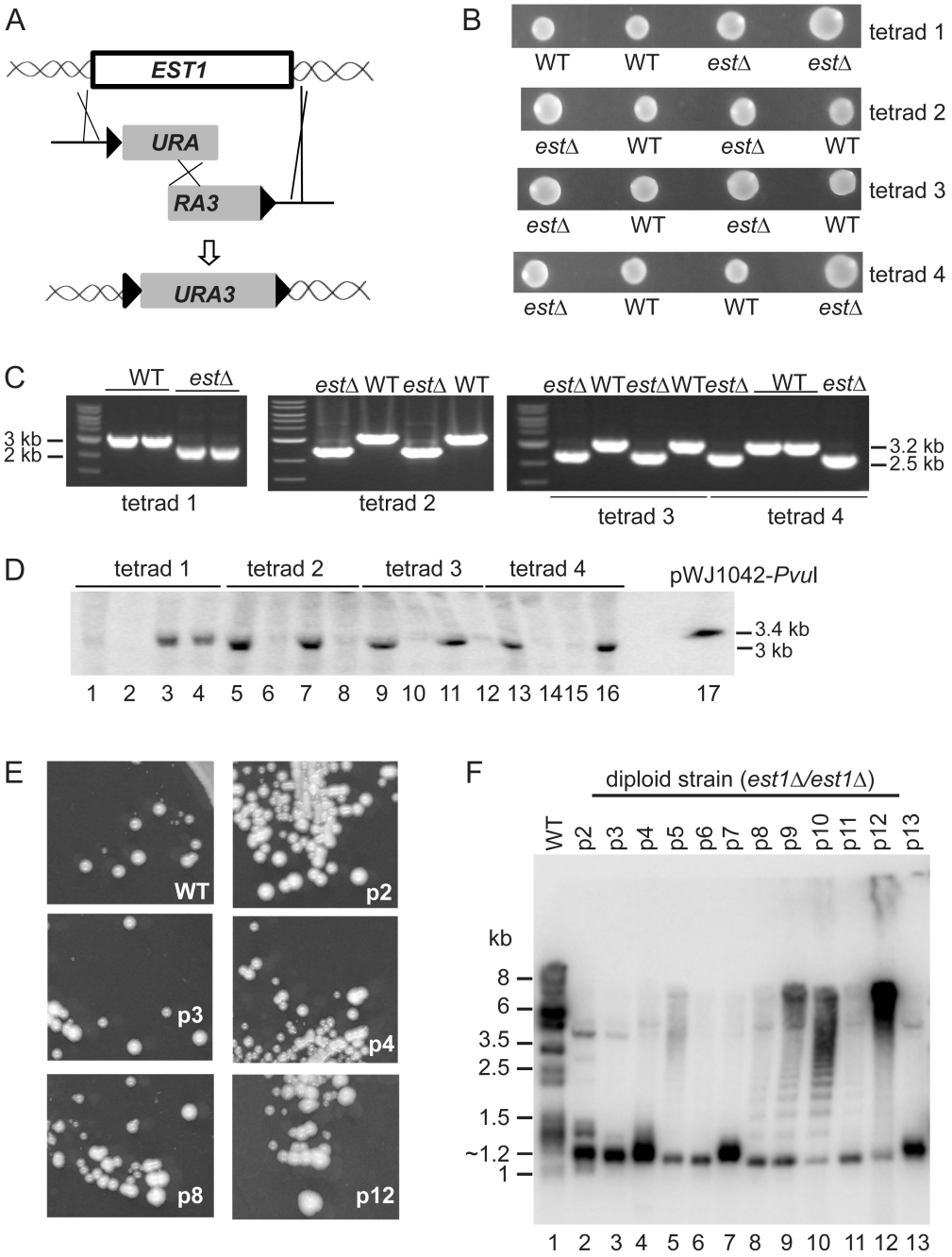


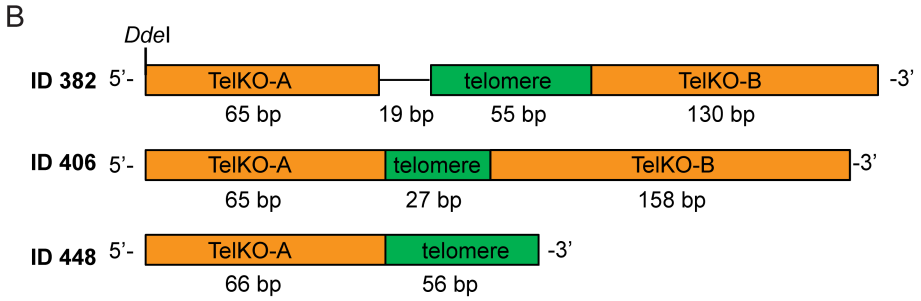
Figure S3. Deletion of the *EST1* gene. (A) Strategy for the replacement of the *EST1* gene with the kl*URA3* cassette. The *EST1* deletion cassette was constructed with 45 bp homologous sequences at the ends. In order to facilitate the correct targeting and gene replacement, we used the ´´split-marker´´ approach, in which three-way recombination occurs to simultaneously insert and assemble the marker gene. The up- and downstream fragments overlap with 452 bp. The 143 nt direct repeats (indicated by black arrow heads) will allow subsequent pop-out of the *URA3* gene. (B) Four tetrads were micro-dissected on YPD plates. Each tetrad contains two wildtype spores (WT; *EST1*) and two knockout spores (*est1*Δ). (C) Genotype analysis of tetrads. PCR using primers annealing 0.5 kb up- and downstream of the *EST1* gene, respectively, results in the WT *EST1* locus showing a 3.2 kb amplicon band, while the replacement by the *URA3* marker generates a 2.5 kb band. (D) Southern blot hybridization of *BamH*I-treated DNA with a *URA3*-specific probe. The insertion of the *URA3* marker at the *EST1* locus is visible at 3 kb, while the WT strains did not hybridize with the probe, as expected. *Pvu*I-cleaved plasmid pWJ1042 containing the *URA3* marker served as a hybridization control (lane 17). (E) Close-up of diploid *EST1* KO colonies (*est1*Δ/*est1*Δ) from the plates shown in Figure4A. (F) TRF assay of diploid *EST1* knockout (*est1*Δ/*est1*Δ) survivors derived from tetrad 2. Genomic DNA from p2-p13 (lanes 2-13) and WT (lane 1) was digested with *Hind*III and Southern blot hybridization performed with a telomeric probe.

A

ALT strain

5'-TTGTTTATTCGATGTTGTTGATATTATGGTAGTGTGGTTTCTATATTTAGGGAGTCTGAGGTTG
GTTATTGTTTGTGGGAAAAGGATAGTTGGTCTGGGTGAGGATATTCTGTTTATGTTTATGTTTAC
GTTTATCTTTGTTTGTTTGTTTATTTGTGTGTTTGTTTTTGTTTGTGTATGTTGGAGGTATTTGTTG
TTGATGGTGGTGTTGTTTTTTTGTCTTTGTGTTTTTTTTCTGGGAGGTGGTTGGTGGATCTAAGA
TTTGTATAGTCTGGGTAATGTATGTCATGGGGTACGAGAAAATGTTGTCTTGGTGAAATT
GTCTGGGTGTCTGGGTGTGTCTGGGTGTCTGGGTGTCTGGGTGTCTGGGTGTCTGCCCCCCCCCC-3'

B



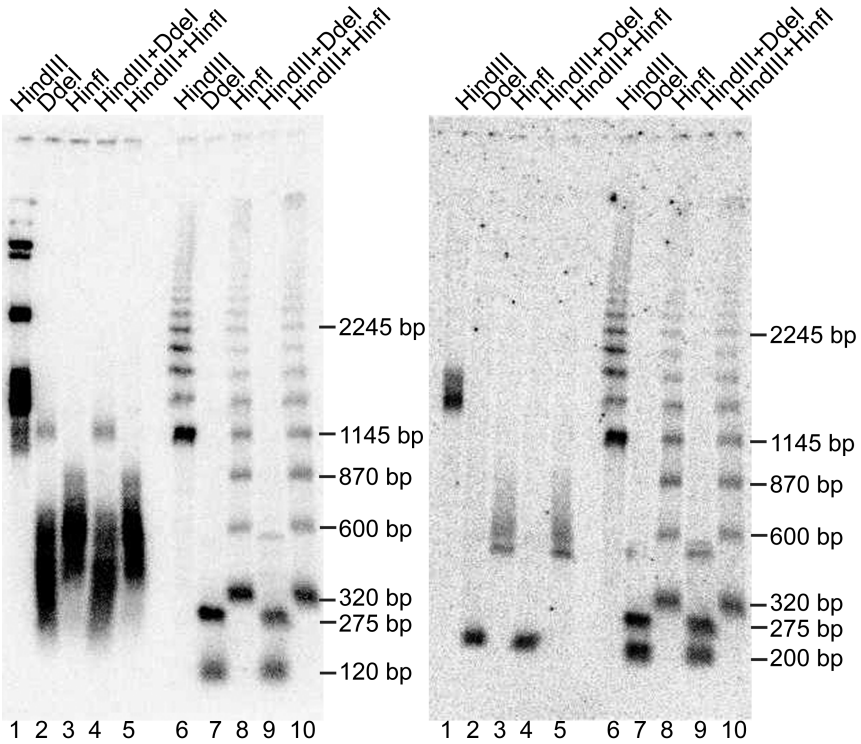
C

ID 382: 269 bp
5'-TAAGATTTGTATAGTCTGGGTAATGTATGTCATGGGGTACGAGAAAATGTTGTCTTGGTGAAATT
TTAGGGGAGCGCGATGCGGGGGTGTCTGGGTGTCTGGGTGTCTGGGTGTCTGGGTGTCTGG
GTGTGTCTGGGTGAGGATATTCTGTTTATGTTTATGTTTACGTTTATCTTTGTTTGTTTGTTTATTT
GTGTGTTTGTTTTTGTTTGTGTATGTTGGAGGTATTTGTTGTTGATGGTGGTGTTGTTTTTTTGTCT
TTGTGTTTT-3'

ID 406: 250 bp
5'-TAAGATTTGTATAGTCTGGGTAATGTATGTCATGGGGTACGAGAAAATGTTGTCTTGGTGAAATT
GTCTGGGTGTCTGGGTGTGTCTGGGTGAGGATATTCTGTTTATGTTTATGTTTACGTTTATCTTTG
TTTGTTTGTTTATTTGTGTGTTTGTTTTTGTTTGTGTATGTTGGAGGTATTTGTTGTTGATGGTGGTG
TTGTTTTTTTGTNTTTGTGTTTTTTTTCTGGGNGGTGGTTGGTGGNTCTAA -3'

ID 448: 122 bp
5'-TAAAGATTTGTATAGTCTGGGTAATGTATGTCATGGGGTACGAGAAAATGTTGTCTTGGTGAAATT
GTCTGGGTGTCTGGGTGTGTCTGGGTGTCTGGGTGTGTGGGTGTCTGGGTGTGTCT -3'

Figure S4. Telomere-PCR and cloning of ALT telomeres. (A) Terminal sequences of the *tlc1*$^{-}$ ALT strain obtained by telomere-PCR. Telomeric sequences are highlighted in green. Green box: The interstitial telomeric sequence (ITS), which is flanking the subtelomeric TelKO element. An 8 bp stretch of telomeric sequence (underlined) is also present inside the TelKO element. The C-tail is indicated in the 3' end. The same sequence was obtained from the WT strain, except with a much longer telomeric sequence. (B) Structural organization of three isolated clones from the *Dde*I sub-genomic library. In clones ID 382 and ID 406 the telomere-proximal TelKO-A and telomere-distal TelKO-B parts of the subtelomeric TelKO element are flanking a stretch of telomeric sequences (green). Clone ID 382 contains 19 bp of unidentified sequence, where only smaller parts match to various places in the *N. castellii* genome in a BLAST search. Since these clones are derived from *Dde*I-fragments, their sequences lack dCTP at 5' end (*Dde*I cleaves at 5'- C^TNAG -3'). Therefore, the TelKO-A element originally contains a 66 bp long DNA segment. (C) DNA sequences of clones ID 382, ID 406, and ID 448, respectively. Telomeric sequences (green highlight), non-defined (gray letters).



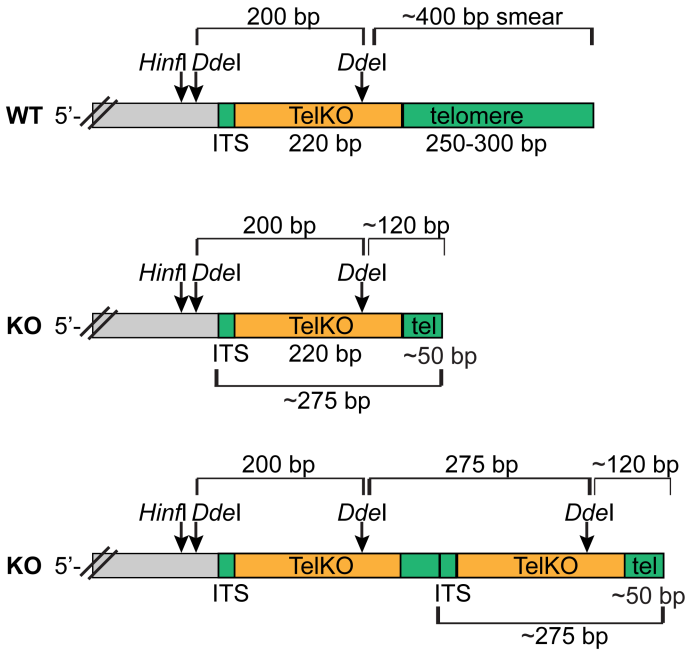


Figure S5. Confirmation of the telomeric structure in *tlc1*⁻ ALT strains by TRF assay with analytical restriction enzymes. The same TRF membrane was first hybridized with a telomeric probe (left) and subsequently stripped and re-hybridized with a probe specific for the subtelomeric TelKO element (right). WT strain (lanes 1-5) and an ALT strain exhibiting the ladder pattern (lanes 6-10). The restriction enzymes used in single versus double digestions are indicated at the top. For convenience in the interpretation, the schematics from Figure 7 is added below.
Left image, hybridized with telomeric probe: *Hind*III digestion generates several telomeric bands between 1-6 kb. Both *Dde*I and *Hinf*I cut telomere-proximal of *Hind*III and collect all telomere fragments into short smears centered at ~400 bp and ~600 bp, respectively (lanes 2-3). This is confirmed in the double digestions where *Hind*III band pattern is displaced by the short terminal *Dde*I/*Hinf*I smears (lanes 4-5). Note that in the ALT strain, *Hinf*I digestion therefore results in a shiftdown of the ladder pattern to shorter ladder fragments, with the shortest fragment of 320 bp

representing a telomere with a single TelKO element (lanes 8 and 10). *Dde*I generates the specific ~275 bp band that is only observed in the ALT strain having the ladder pattern (lanes 7 and 9), and which is demonstrating the repeated TelKO elements. In addition, *Dde*I generates the 120 bp terminal band containing the shortened ~50 bp telomeric sequence.

Right image, hybridized with the TelKO probe: Notably, in the WT strain, the TelKO-specific probe only hybridizes with one of the *Hind*III fragments, indicating that some telomeres lack this particular subtelomeric region (lane 1). *Hinf*I generates a smear in WT, while the KO exhibits the ladder pattern which is shifted down compared to the *Hind*III ladder (lanes 8 and 10). In both WT and KO strains, *Dde*I releases a 200 bp fragment containing the TelKO region (lanes 2, 4, 7 and 9). Notably, in the ALT strain, *Dde*I also generates the ~275 bp band that is specific for the ladder pattern strains, and which is derived from the repeated TelKO elements. From the sequencing data we could conclude that this band corresponds to the ~275 bp *Dde*I fragment encompassing an internally placed telomeric sequence stretch flanked by upstream and downstream regions of TelKO elements. Significantly, this exact structure was also retreived in the clones obtained in the subcloning experiment. Taken together, this TRF assay confirms the structure obtained from the sequencing data.

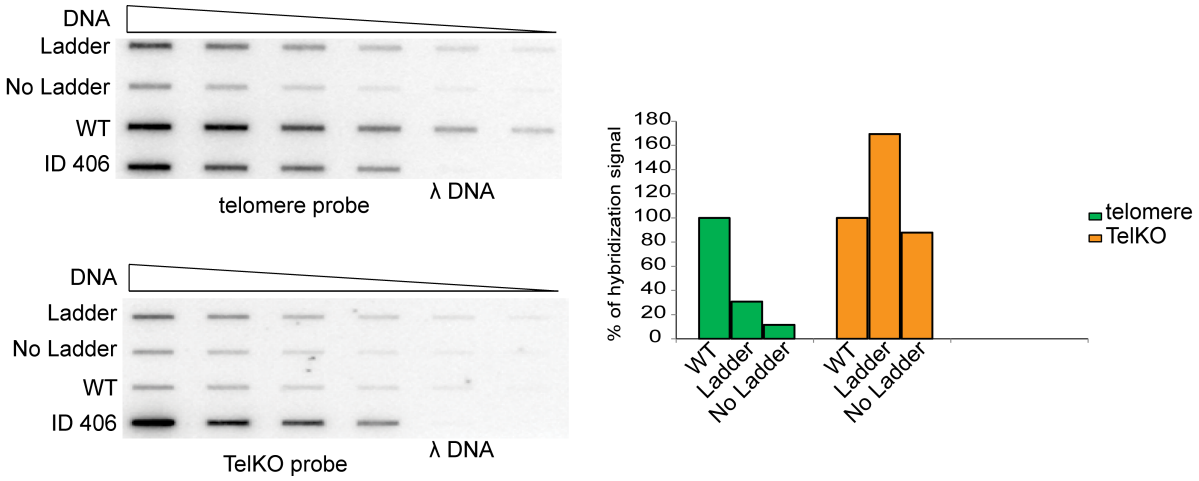


Figure S6. Quantification of TelKO elements in the *N. castellii* genome. Slot blot hybridizations with a telomeric probe and a TelKO-specific probe, respectively. Genomic DNA extracted from diploid (*tlc1*$^-$/*tlc1*$^-$) survivors from p11, having ladder pattern and no ladder, respectively. The WT type strain Y-12630 (WT) was included for normalization. Two-fold serial dilution of 256 ng DNA was blotted on the membrane. Plasmid DNA of the clone ID 406 (8-1 ng) was spotted as a positive hybridization control, and 250 ng of λ DNA was used as a negative control. Hybridization signals were quantified, normalized against λ DNA and then the value of WT DNA. Relative percentage values were plotted in the diagram (WT DNA was set to 100 %).