4 RESULTS

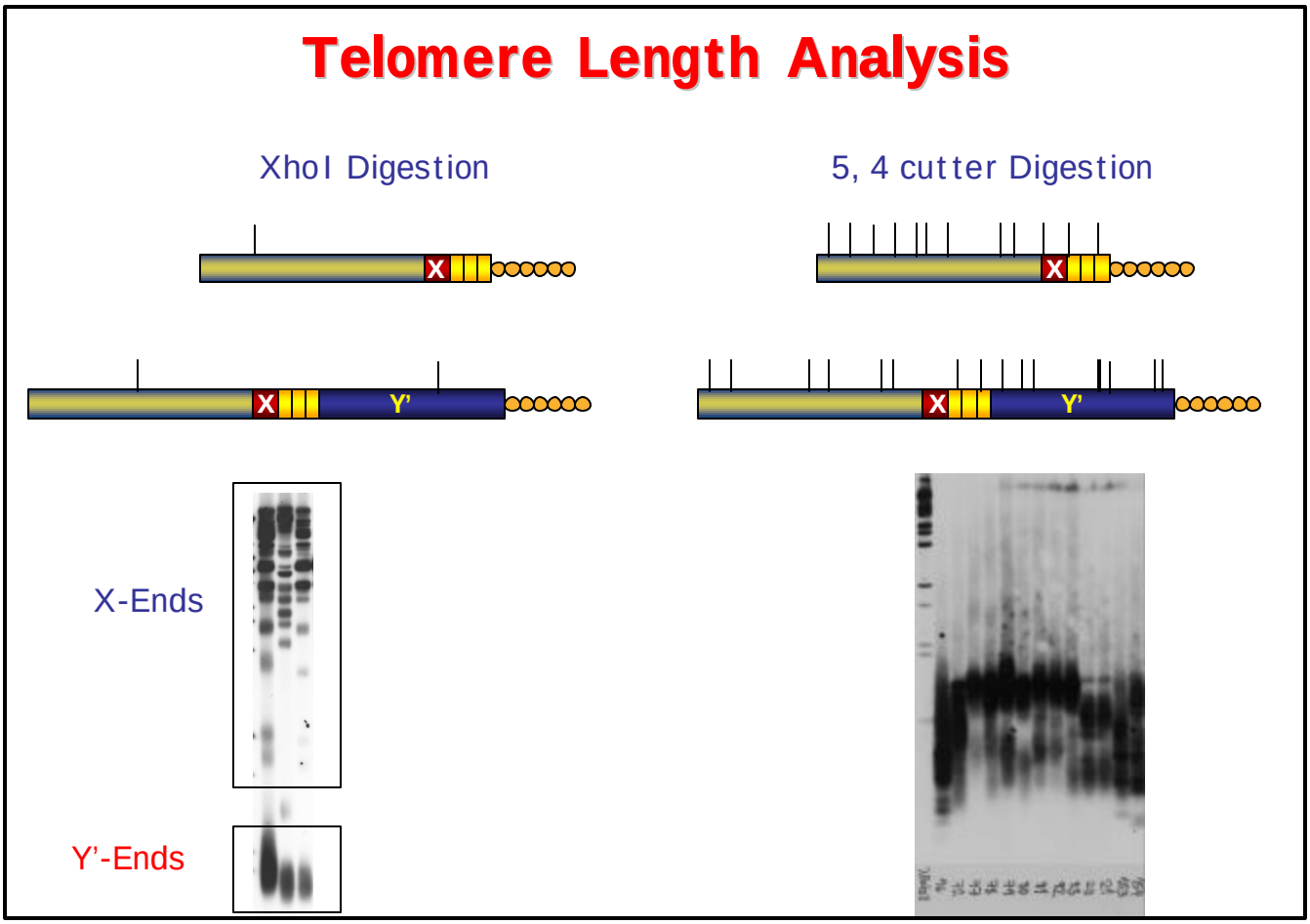
1.1 TELOMERE LENGTH VARIATION ANALYSIS

Previously, subtelomeric variation was characterized for 112 different strain isolates of the *Saccharomyces sensu stricto* complex. Electrophoretic karyotypes exhibited the distribution and variability of the Y' elements within the isolates (except *S. bayanus*). Phylogenetic inferences made from locations of these Y'elements made it possible to determine the most likely origin of Y' elements. Overall, *S. cerevisiae* strains displayed a large number of chromosomes harbouring Y' elements, while the wild-type Far East Isolates of *S. paradoxus* presented very low abundance of this telomeric associated sequence (TAS)[14]. Liti *et al*, found a Far East Isolate, N-44 (Figure 5, lane 26), which is Y' deficient.

Telomere length was assessed in these *S. paradoxus* isolates and compared to *S. cerevisiae* trains. A collection of 40 *S. cerevisiae* strains and 48 *S. paradoxus* strains were used for this analysis. Two approaches were utilized for the assesment of telomere length. The first was the *XhoI* digestion, the second, a mixture of 5 enzymes that cut every 4 base pairs. Two different approaches were utilized in order to obtain a clearer definition of the actual size of the telomere. *XhoI* digestions shed information only about Y' ends in yeast. Y' elements are generally present only in 2/3 of the total telomeres in the *Saccharomyces sensu stricto* complex. To complete the information obtained from the *XhoI* digests, the 5,4 cutter digestion technique was followed. This approach was designed to digest the whole of the yeast genome except within the telomeric tracts. This second approach was needed also because of the presence of polymorphisms in the *S. paradoxus* genomes, specially for the Far East *S. paradoxus* isolates which have very low Y' copy number.

DNA from each strain in the collection was extracted using the Phenol-Chloroform tecnique. The extracted DNA was digested overnight at 30°C either with *XhoI* or with 5 different restriction endonucleases (*AluI, HinfI, HaeIII, MspI, MboI*). The *XhoI* digests were run on 0.9% agarose gels, while the 5,4 cutter digests were run on 1.3% agarose gels. Southern Blot analysis followed, using a telomeric probe (pKK38). Theoretically and in the practice, results obtained from both digestions revealed similar telomere lengths for the different strains. In some of the 5,4 cutter digestion gels, DNA was not completely digested. Yet, overall, one could compare results between the two techniques.

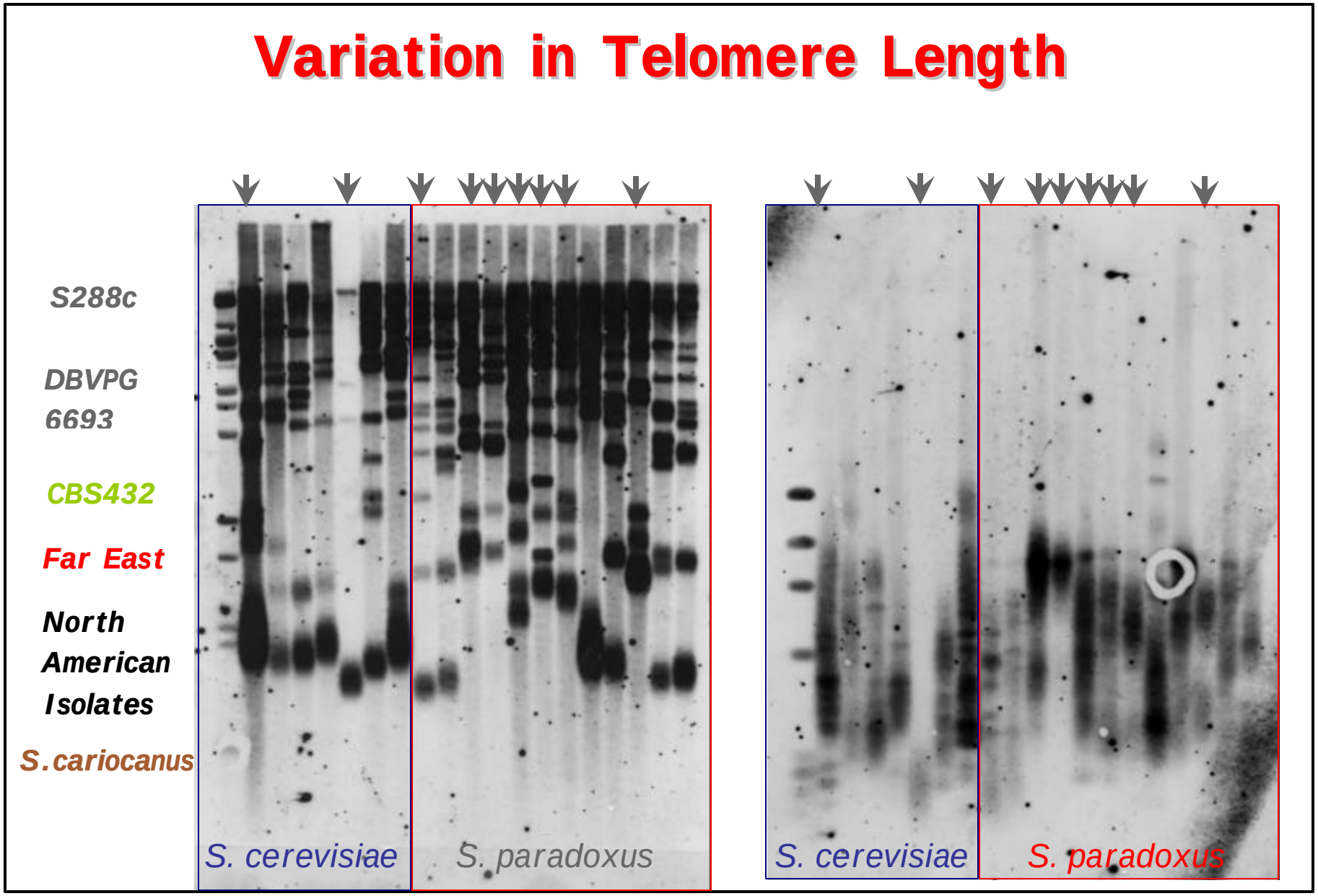
Figure 8 Telomere Length Analysis utilizing two different approaches: XhoI digestion for Y' end information, and a mixture of 5, 4 cutters to verify the XhoI results



Telomere length correlate with geographic distribution in both *S. cerevisiae* and *S. paradoxus* strains. These patterns, in addition with the ones obtained for subtelomeric sequences, may yield interesting clues as to the evolution of the inter-species mechanisms that control telomere length. Overall, telomere length homogenization between the *S. cervisiae* geographical isolates was observed. In contrast, the telomere lengths exhibited by the different *S. paradoxus* isolates were much more heterogeneous. These observations correlate directly with the assuption that a decrease in genetic variation within the different *S. cerevisiae* isolates is a consequence of its intensive selection in human related activities.

The most important size differences between the *S. cerevisiae* isolates was observed between two strains, the S288c isolate, which is the *S. cerevisiae* sequenced strain, with long telomeric tracts, and the 6693 isolate (from a Belgium brewery) which exhibited short telomeres. For the *S.paradoxus* isolates, telomere length varied among the different geographical isolates. The shortest telomeres were

Figure 9 Southern Blot Analysis of XhoI digestions and 5, 4 cutter digestions.



observed in the sequenced strain of *S. paradoxus*, CBS432. Interestingly, the longest telomeres were present in the Far East isolates and in the only *S. cariocanus* isolate. Similar telomere length was observed between all the North American Isolates. The discovery high telomere length variation within the *S. paradoxus* strains can be used to question the evolution of telomere length control mechanisms.

1.2 TELOMERE TAG EXPERIMENT

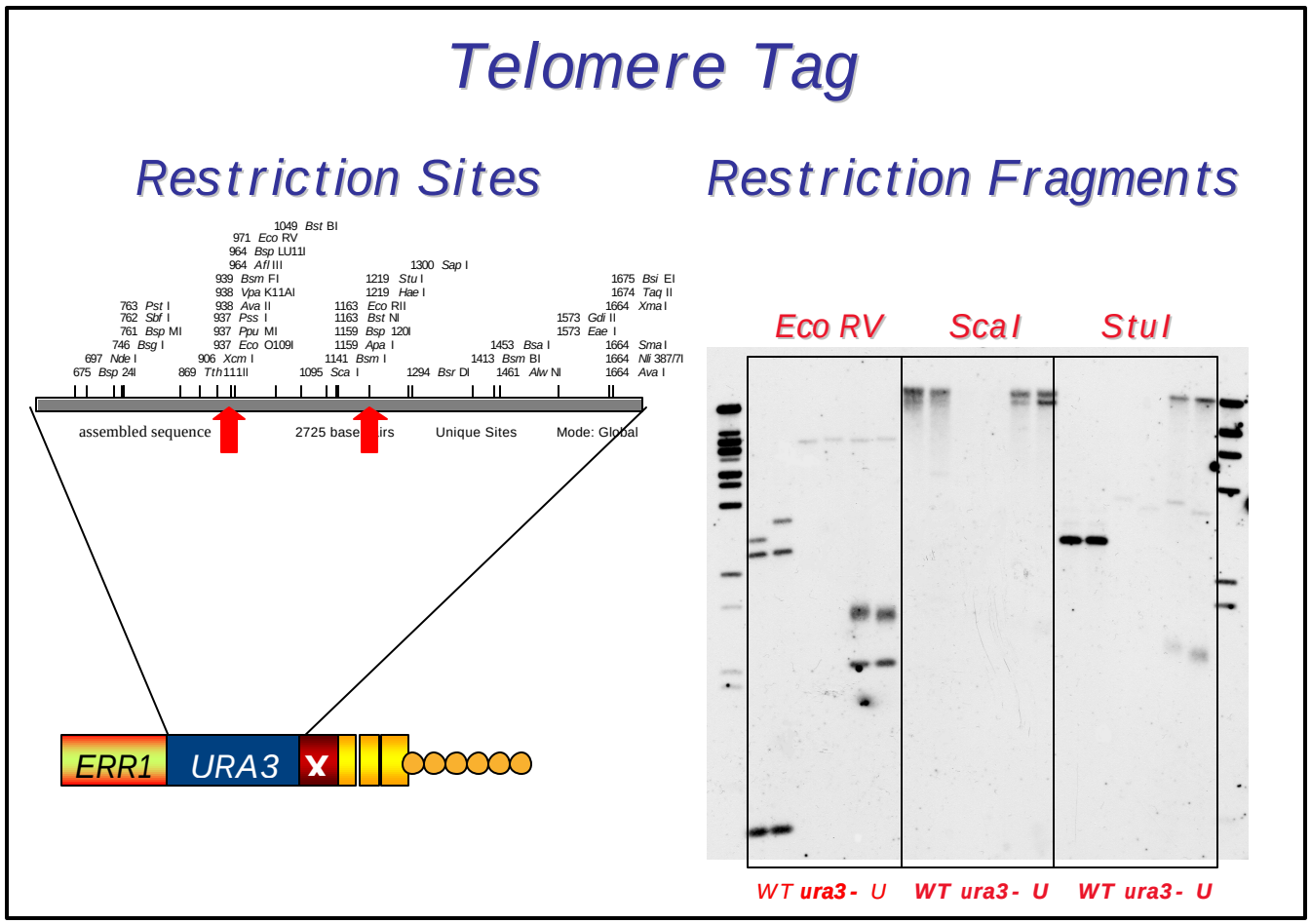
A new approach was taken by tagging one chromosome end. The telomere tag is an artificial chromosome end that has a standardized number of telomeric repeats. By inserting this tag into a single chromosome, one can follow the processes of telomere shortening or lengthening with each round of replication . The telomere tag was designed previously by Louis *et al*. The telomere tag construct contained a fragment of the ERR1 telomere associated sequence, which is a single copy gene in *S. paradoxus* found on chromosome XV. Secondly, it contained a *URA3* marker, for screening purposes. The inclusion of the CoreX sequence was to make this artificial telomere more like a natural end. (it also may be involved in many of the telomere length control mechanisms). Finally, 200 bp of telomere repeats were added. The total length of the construct was 2.7 kb.

Two *S. paradoxus ura3*$^{-}$ strains were transformed with this telomere tag. The selected isolates were the CBS432 sequenced strain, which had been caracterized for having very short telomeres, and the N-44 Far East isolate which had very long telomeres. It was inferred that once the isolates had uptaken the telomere tag solely on chromosome XV, the nuclear telomeric machinery would detect this unusual chromosome end length and either add or degrade certain number of repeats. Generating a telomere tag which was had the species-especific telomeric length.

After screening 250 positive transformants, two strains were isolated harbouring the insert in the correct position (chromosome XV). The DNA from these two strains was extracted and digested with 3 different restriction enzymes: *ScaI, StuI* and *EcoRV*. These three enzymes had only one restriction site within the *URA3* marker, generating a known length fragment.

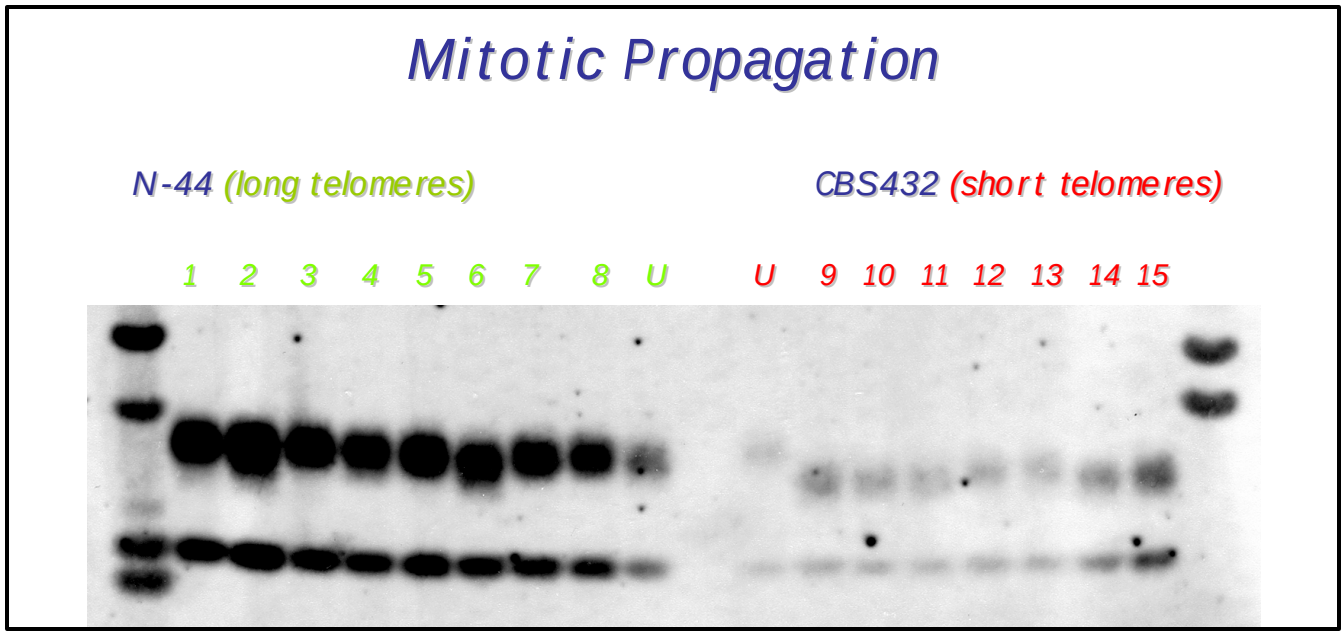
Immediately, Southern Blot Analysis continued. Only to discover that the fragments generated from this autoradiography were the same size as the construct size, 2.7kb. Concluding that the cells hadn't had enough buffer time of coevolution, mitotic propagation of the cells followed. The two telomere tagged strains were propagated on YPD plates for 200 generations before DNA extraction. For this second round of Southern Blot Analysis, the DNA was digested with only *EcoRV* and *StuI*. These two enzymes generated an easier band pattern to follow.

Figure 10 A 2.7 kb artificial telomeric end. The construct was designed and inserted into a plasmid. This plasmid was used to transform two S. paradoxus strains. On the left, determination of the different restriction sites within the URA3 marker. On the right, southern blot analysis of the telomeric restriction fragments generated by 3 different enzymes.



Surprisingly, results from the mitotic propagation indicated that the cells from the N-44 strain had added telomeric repeats, while the telomeric ends of the CBS432 strain had shortened. Thus, the telomeric regulatory mechanisms (telomerase-nucleolytic degradation) had acted upon these exogenous telomeres to reestablish their species-specific length. Although the length observed in the mitotic propagation was not the length caracterized previously by the telomeric length analysis experiment; this discrepancy could be explained by acknowledging that telomerase does not act on all chromosome ends (16 chromosomes=32 chromosome ends in yeast) at each round of replication. Probably the on-going propagation of these strains for another 100-150 generations would give a higher probability of telomerase activity over most ends.

Figure 11 Mitotic Propagation of both *S. paradoxus* strains harbouring the telomere tag. Lanes labeled 1-15 contain the EcoRV digestion of the propagated cells (200 generations). Lanes labeled U are the unpropagated "standardized" telomeric length.



To observe if meiotic recombination had a clear effect on telomeric length control mechanisms, a second experiment was designed. Sporulation was induced in both propagated strains. Comparisons between the mitotically propagated colonies with the meiotically induced colonies showed no differential effect. Recombination had not altered telomere length in these strains.

Figure 12 Meiotic Effect of both *S. paradoxus* telomere tag strains. Southern Blot Analysis of EcoRV digestions. Blue rectangles are the mitotically propagated colony from whitch the four spores were taken (red rectangles)

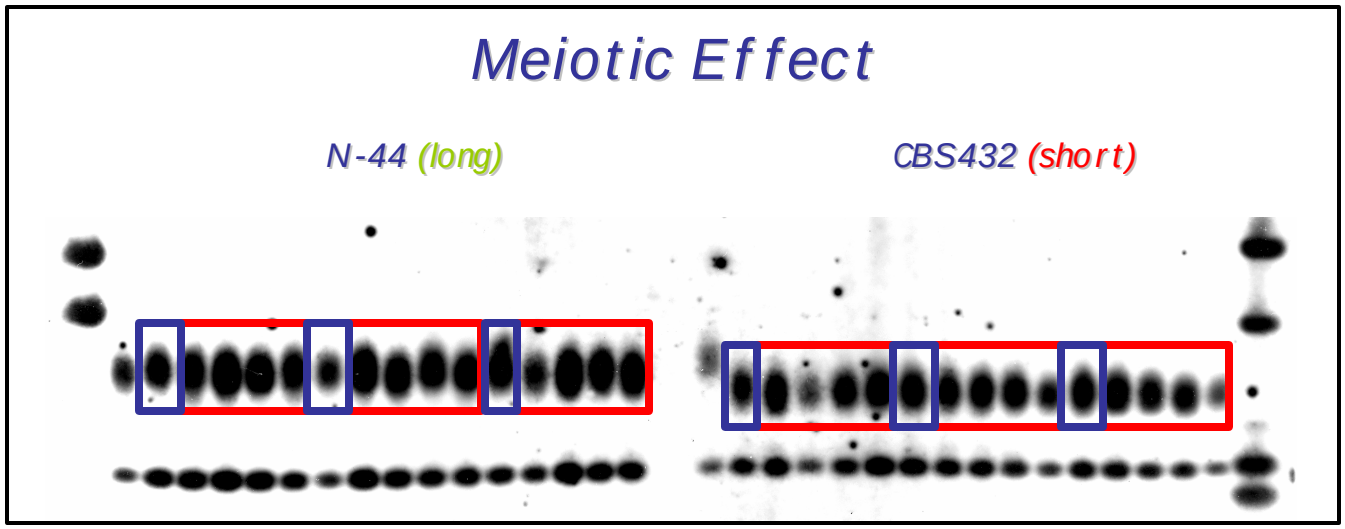
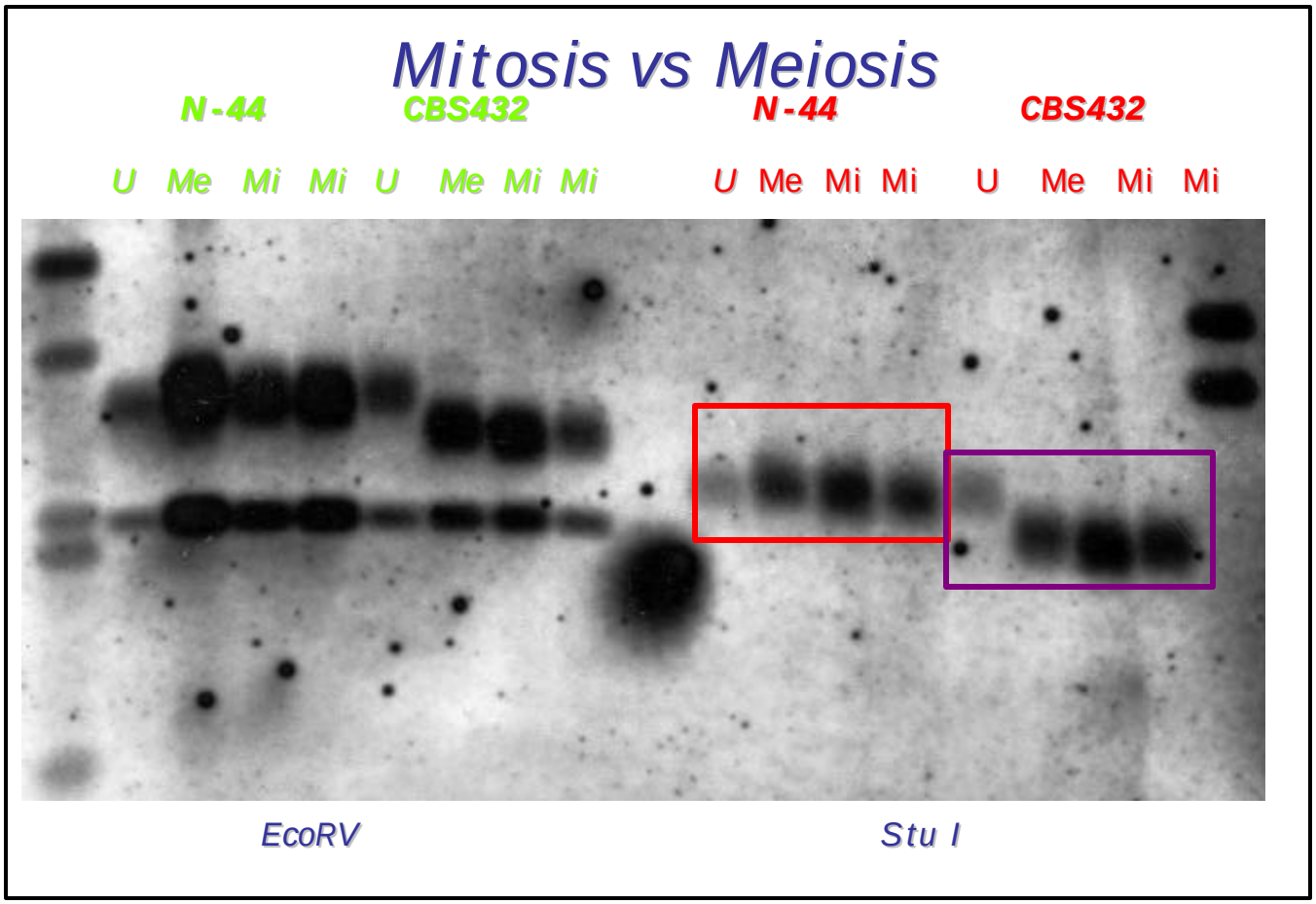


Figure 13 Mitotic vs Meiotic effects on telomere length. Lanes labeled U are the unpropagated "standardized" cells. Lanes labeled Me are the meiotically propagated cells, lanes labeled Mi are the mitotically propagated cells



1.3 ALTERNATIVE LENGTHENING OF TELOMERES (ALT) ANALYSIS

To further understand telomerase-independent mechanisms for telomere maintenance in yeast, strains of *S. paradoxus* lacking *EST2*, the gene encoding the catalytic subunit of telomerase, were created. Two types of survivors arise, overcoming the resulting senescence, and maintain their telomeres by recombinational-based methods. The complexity of generation and maintenance of such survivors suggest the interaction of multiple gene products, such as helicases, nucleases, replication proteins, chromatin factors, among others. To test the effects of the genes involved in the two survival pathways, disruption experiments were carried out.

The gene of interest was disrupted in a diploid heterozygous EST2/est2::KanMX strain. This method involves the PCR amplification of a marker gene (*KanMX*, *HYG*, *URA3*) using primers with short flanking homology to the gene of interest. The diploid yeast strain is then transformed using this construct and the gene of interest is disrupted. Cells can be easily selected on selective media. Such disruptants were then verified using colony PCR .

Sporulation and disection of the heterozygous diploids allows selection of haploids that have the *EST2* disruption. Perfect Mendelian segregation can be observed in the 4 spores (haploid) of each ascus. Two harbouring the *EST2* activated gene and the other two the disrupted *EST2* gene. The diploid colonies (self-mating diploids) which arrise from each disected spore can then be propagated in YPD media. Only the disrupted colonies were of interest for propagation. These were streaked for 75 to 100 generations or until crisis. The assumption of the generation time is that two days growth at 30°C is approximately 25 cell generations. Post-senescent survivors appeared and were propagated further.

The disruptions were carried out in two *S. paradoxus* strains. The first strain that was mutated, was the sequenced strain CBS432 with natural short telomere variation, whilst the second disuptants came from the N-44 Far East isolate which presented long chromosomal ends. The CBS432 *est2* strain (YGL135) was utilized for the senescence experiment. Knowing that the

N-44 *est2* (YGL54) strain is Y' deficient, it became a very interesting strain in which to analyse ALT mechanisms.

In the absence of telomerase, two recombinational-based pathways arrise in post-senescent survivors. The type I survival pathway amplifies Y' elements, while type II abruptly amplifies the telomeric repeats. The type I pathway is dependent on *RAD51*, while the type II mechanism of telomere maintenance relies on the activity of *RAD50*[29]. The YGL54 strain naturally lacks Y', consecuently abolishing the type I pathway option for survival. The only viable option for this *est2* strain would be to undertake the type II TG_{1-3} amplification pathway. The exonucleolytic activity of *RAD50* can be disrupted through gene *knock-out* techniques. By inactivating this gene, the type II pathway would be abolished and only type I survivors should arise. Thus, an *est2 rad50* double mutant was created to prove the accepted hypothesis. These transformants were propagated for 200 generations, allowing enough time for senescence and survival appearance. In a typical experiment, 8-10 single, small colonies of each strain were streaked on YPD plates, incubated for 48 hours at 30°C, examined for growth phenotypes, and photographed (~25 generations). Then, single colonies from the first passage were restreaked and analyzed similarly (~50 generations). This process was repeated 6 more times.

Growth phenotypes differed between the WT, single mutants and the double mutant. The *rad50/est2* strain had a more severe growth defect than the *rad50* and *est2* mutants alone. The double mutant showed a period of slow growth between 75 and 125 generations, but regained a steady growth rate, indicative of the generation of stable survivors. Yet colony size remained smaller than the WT and single mutants after 200 generations. Overall, *est2* single mutants grew less than the single *rad50* mutants. Colony size of the single mutants was similar to the WT strain after 200 generations of propagation. *Rad50* single mutants do not enter a senescent phase, yet they undego growth retardation as an effect of the inactivation of a key protein in DNA damage repair mechanisms.

Figure 14 Propagation after 75 generations of YGL54 (Y' deficient) strain. Clockwise: WT strain, est2 single mutant, rad50/est2 double mutant and rad50 single mutant.

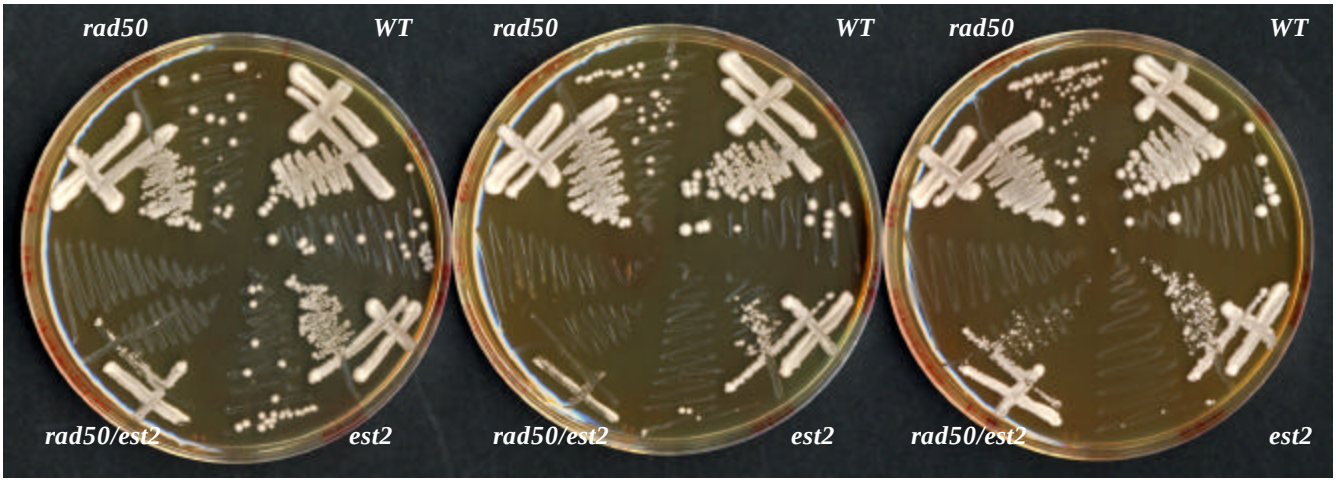
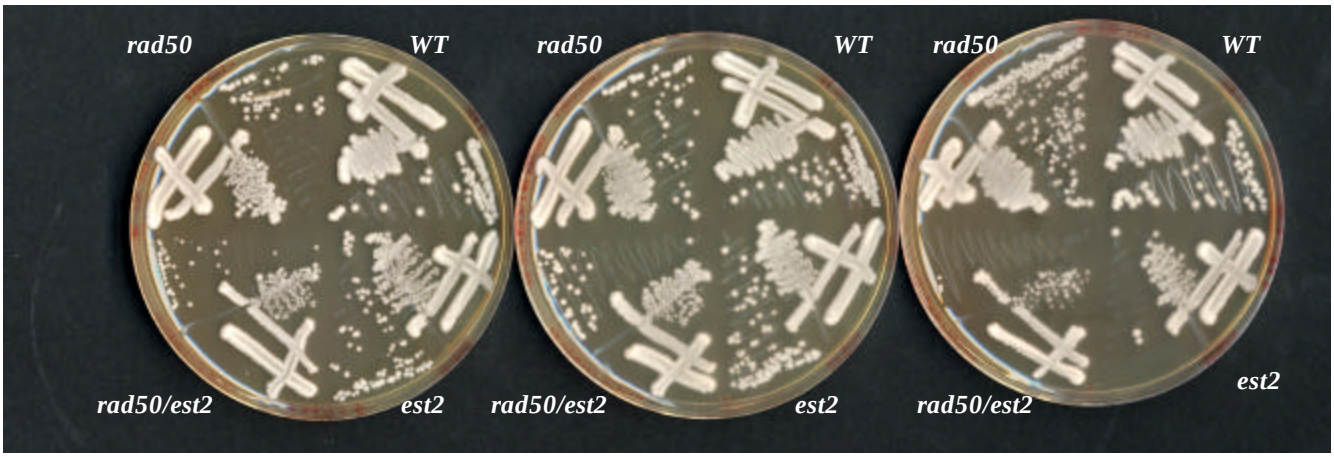


Figure 15 Propagation after 200 generations of YGL54 (Y' deficient) strain. Clockwise: WT strain, est2 single mutant, rad50/est2 double mutant and rad50 single mutant.

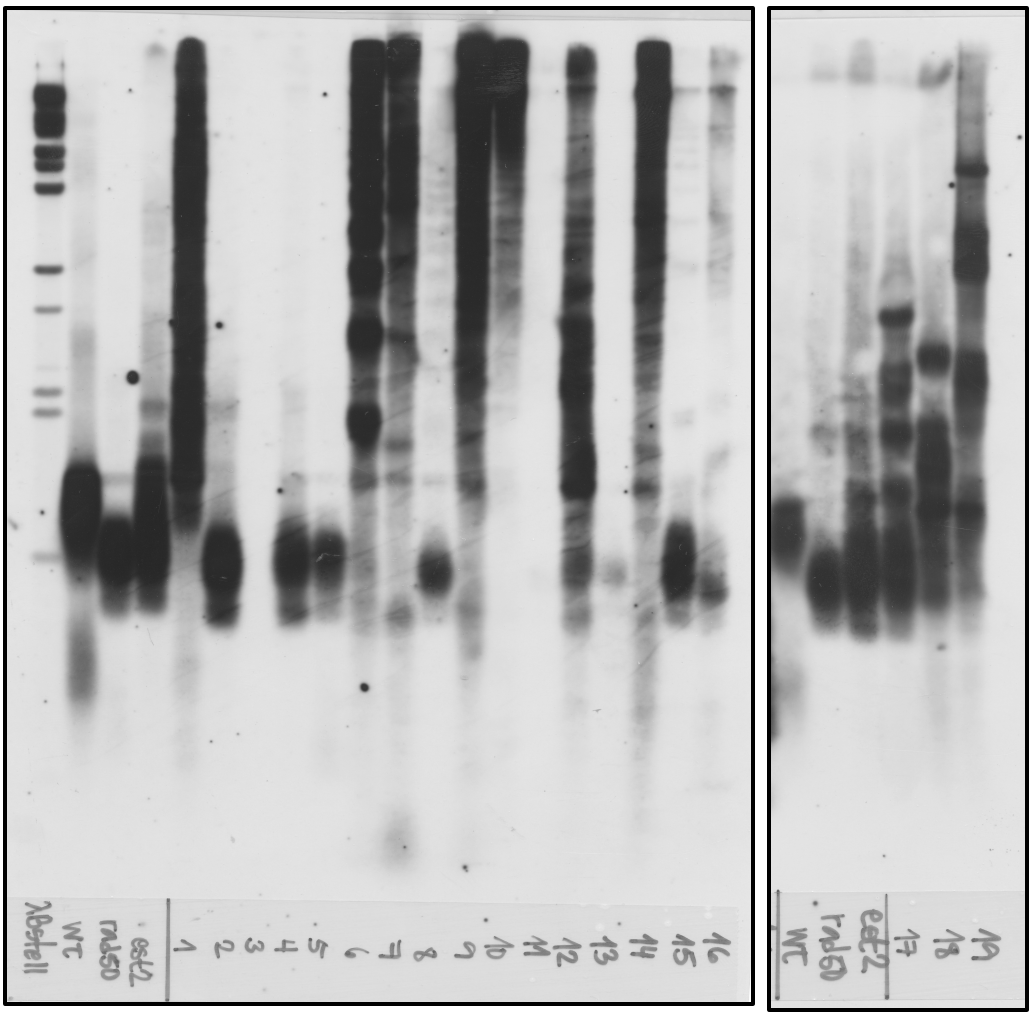


After 200 generations, the DNA from the strains were digested with *XhoI*, separated by gel electrophoresis under standardized conditions and subjected to Southern blot analysis with a telomere-specific probe. The telomeric probe (pKK38) produced two very distinct band patterns, derived from the hibridization to telomeric repeats. Previously, southern blot analysis had revealed the naturally long telomere of the wild-type YGL54 strain. Both *rad50* and *est2* single

mutants were found to have shorter telomeres than the WT, suggesting the amplification of a subtelomeric repeated sequence.

The 16 independent double-mutant survivors choose two distinct mechanisms for telomere maintenance. The first pathway, taken by 44% of the survivors, generated a fragment pattern similar to the *est2* and *rad50* single mutants (similar telomere length). The second group of survivors, comprising 56% of the total, generated a ladder-like band pattern, suggesting the amplification of long tracts of telomeric repeats, similar to type II survivors. These results clearly state that the correlation between recombination pathway and survivor class may not be absolute, as type II survivors were obtained in a *rad50* mutant strain and type I survivors appeared in a Y' deficient strain.

Figure 16 Southern Blot of *XhoI* digested *S. paradoxus* N-44 Y' deficient strain. WT, *rad50* single mutant, *est2* single mutant and 16 independent *rad50/est2* double mutants. The double mutants undergo two distinct survival mechanisms. Type I-Y' Amplification-Independent pathway, and Type II-Rad50-Independent pathway.

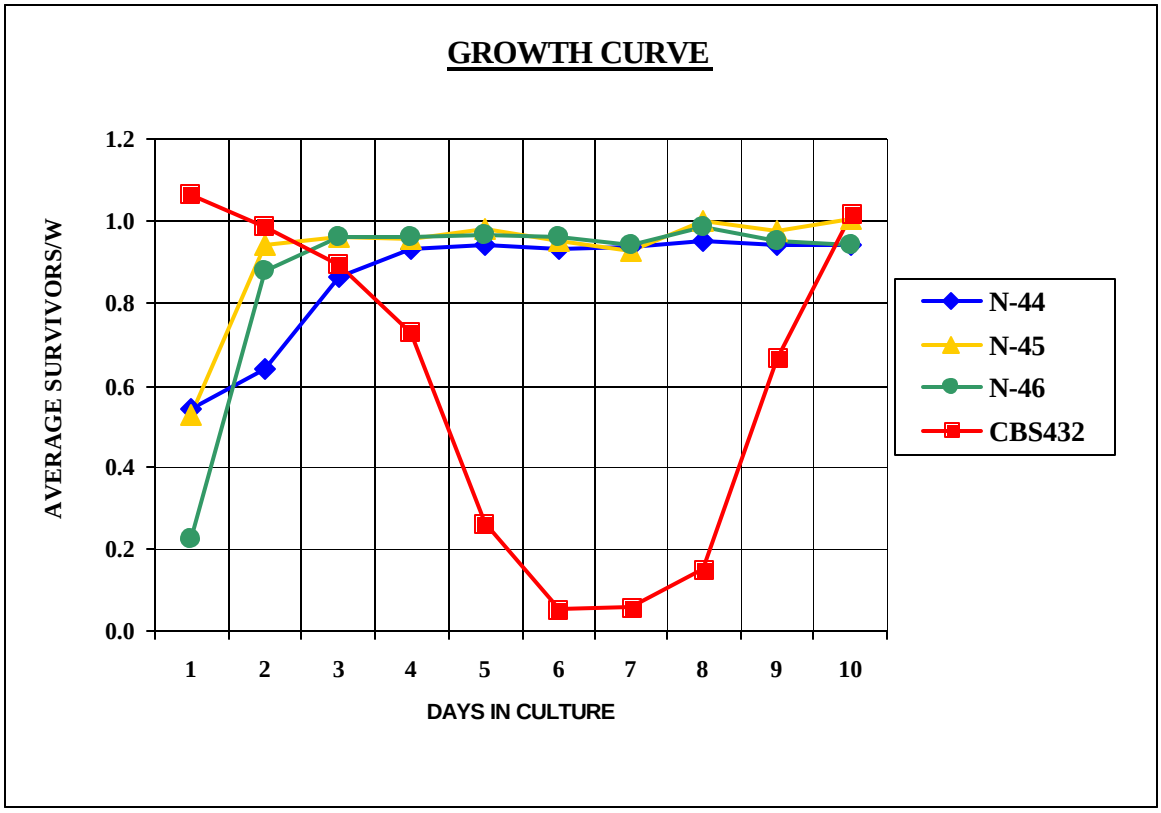


1.4 SENESCENCE EXPERIMENT

Senescence and survival rates were assesed in four *S. paradoxus* strains. The first strain, CBS432 is the sequenced *S. paradoxus* strain, caracterized by short telomeric tracts (400 bp). The second is the Y' deficient N-44 Far East isolate, which has long telomere repeats (800 bp). Two other two were Far East isolates harbouring one and two Y' copies only. These *est2* mutant cells were passaged in YPD liquid media by serial dilution to examine the duration of senescence and survival recovery. Cells taken from thawed stocks were inoculated in 5ml YPD liquid media at 30°C the night before the first reading. The spectrophotometer readings were taken at 600nm, adding only 100 µl of the cell culture and 900 µl of dH_20. This ten fold dilution was required for accurate readings. After the readings, cells were diluted to a standard concentration in fresh YPD media, incubated at 30°C for 24 hrs and then measured cell density in the same manner as described before the following day. This cycle was repeated for 10 days. Using this assay, each day of growth represents up to 10 generations.

The results from the spectrophotometer readings generate speculation. Clearly, the Far East isolates have much more rapid rates of senescence than previously thought. Rather than the usual 60-80 somatic cell generations until senescence, these telomerase negative Far East isolates (N-44, N-45 and N-46), undergo senescence within the first 20 generations. Contrary, the CBS432 strain with shorter telomere senescesed later (60 generations). The faster rate of senescence in the Far East isolates is counter intuitive, as longer telomeres should result in a longer buffer period in the absence of telomerase.

Figure 17 Spectrophotometer UV/VIS readings of telomerase negative *S. paradoxus* isolates



A second yet similar experiment was made to confirm the spectrophotometer results. The strains were obtained from thawed stocks and transformed (*est2* knock-out). Screening for positive transformants followed. Sporulation was induced, and the Mendelian segregation of *est2* and *EST2* spores could be clearly seen. Tightly controlled propagation continued to obtain a senescence time course. This experiment concluded with the same results as the liquid media time cell culture time course. Why is the senescence rate not directly related to longer or shorter telomeres in these strains?

Figure 19 N-44 dissection plate after 2 days growth (20 generations). Bigger sized colonies are the *EST2* spore colonies while the smaller sized colonies are the senescing *est2* cells.

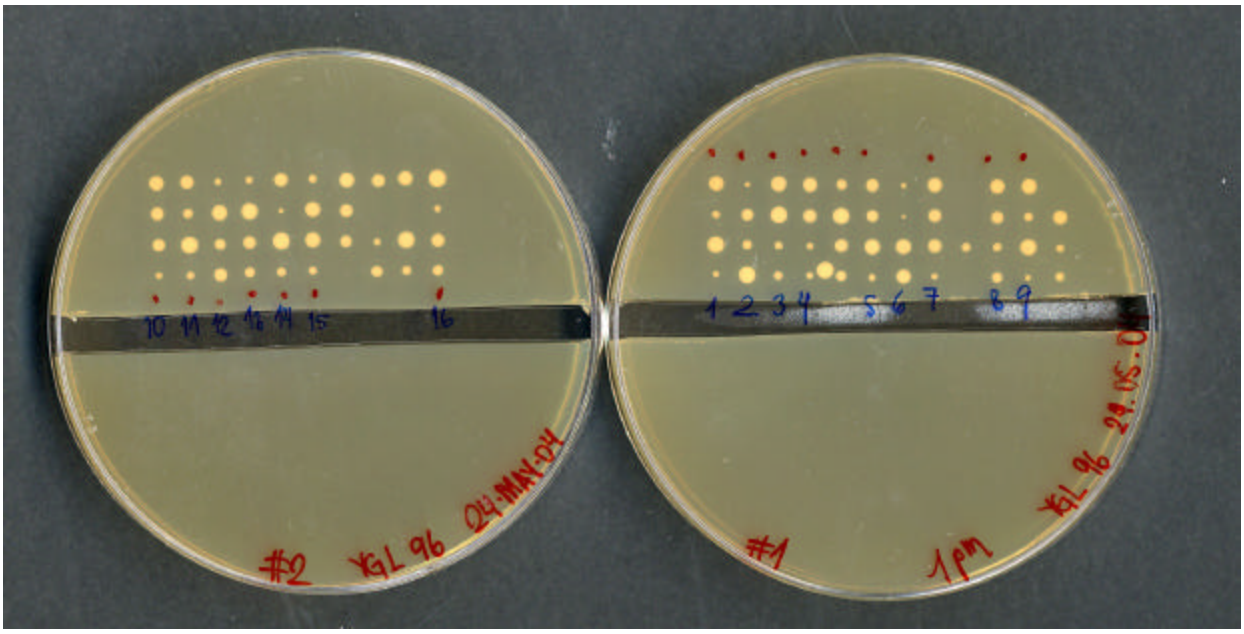


Figure 18 CBS342 Senescence time course. Graph representing colony size area differences between WT and est2 cells. Streaked est2 colonies. After 20 generations on dissection plate, after 40 and at 60 generations one can observe the initiation of senescence