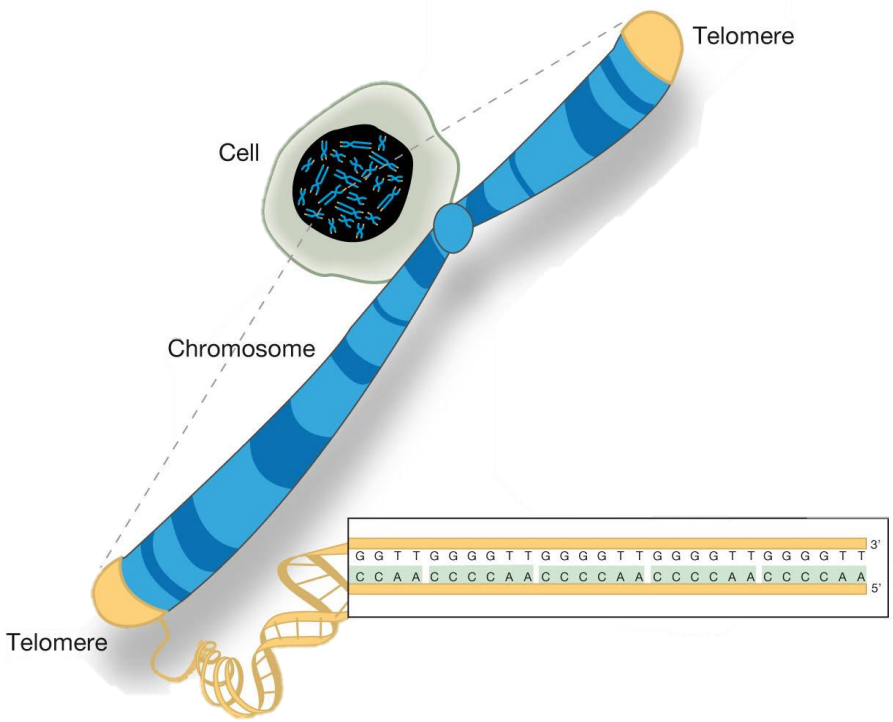
Proteomics and chromatin remodeler genes in *Saccharomyces cerevisiae* survivors



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Abstract

Every year, millions of people die due to cancer. This makes optimization of anticancer therapy and understanding the formation of cancer the interest of many researchers.

Cancer is defined by six hallmarks, one of which is limitless replicative potential. This hallmark can be obtained by the maintenance of telomeres. Cells can maintain the length of their telomeres by activating telomerase, a reverse transcriptase, or alternative lengthening of telomeres (ALT), a recombination-like mechanism.

Saccharomyces cerevisiae (budding yeast) naturally expresses telomerase, but when this is inactivated yeast is no longer able to replicate unlimited and starts to senesce. Some of these senescing cells overcome senescence via the activation of ALT, after which the yeast cells are called survivors. However, it is not known yet what triggers this activation. Therefore, we investigated if the proteome changes during senescence and survivor formation. Furthermore, we investigated if six different chromatin remodeler genes, *ASF1*, *SWI3*, *SNF5*, *SET2*, *SIR2* and *IES4*, are involved in senescence and survivor formation in budding yeast.

To quantify the changes in the proteome an arginine and lysine auxotrophic telomerase negative strain was generated and we found that these changes in the strain did not affect senescence and survivor formation.

After the deletion of selected chromatin remodeler genes in telomerase negative budding yeast, we found that *swi3Δ* and *snf5Δ* mutants cannot be obtained due to sporulation problems. We also found that the *asf1* deletion increases the senescence rate and survivor formation rate of telomerase negative yeast. Furthermore, we found that the deletion of *set2* seems to prevent yeast from going into senescence. And finally, we found that the *sir2* and *ies4* deletions do not seem to affect senescence and survivor formation.

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Introduction

General information

Every year, 8.2 million people die due to cancer and 14.1 million new cancer cases are reported (data till 2012). These numbers are increasing every year [(1)]. Reducing cancer formation has been the main interest in the cancer field these past few years, by focusing on optimization of anticancer therapy and on the understanding of cancer formation. To understand cancer diversity, six main hallmarks of cancer have been reported[(2)]. One of them is limitless replicative potential. Most somatic cells have a finite replicative potential, and because of this they stop growing after a certain number of divisions. This process of reduced cell growth and cell division is termed senescence. However, cancer cells seem to avoid this processes by prevention of telomere attrition, which is a hallmark of aging[(3)].

Telomeres

Telomeres are nonprotein coding DNA sequences at the end of linear chromosomes in eukaryotic cells. The highly repetitive sequences of the telomeres differ between organisms. For example, in *Saccharomyces cerevisiae*, also known as budding yeast and Baker's yeast, telomere length is approximated to 300 ± 75 bp and consists of the repetition of the sequence $G_{2\text{-}3}(TG)_{1\text{-}6}T$ (5' to 3')[(4)]. Further, telomeres are surrounded by proteins that function as a cap to prevent the telomeres from being recognized as double stranded breaks. When this cap is lost, cells will go into senescence or undergo apoptosis, because the loss will be recognized as a double stranded break or because DNA damage is registered due to chromosomes that are now able to fuse to another chromosome. It is proposed that the loss of these protective capping proteins, and with that the increase of senescence and apoptosis, occurs when telomeres become critically short [(3, 5-8)].

In eukaryotic somatic cells, telomeres shorten every cell division due to the end-replication process problem. This problem arises due to the fact that DNA polymerase cannot synthesize a complementary strand de novo or from the 3' to 5' direction on the template strand. DNA polymerase can only make a new strand from a free 3' hydroxyl group. RNA primers provide this structure and therefore make it possible to copy the original template. However, when the RNA primers are removed during the replication process , there is no hydroxyl group at the outmost 5' end for the DNA polymerase to bind. Therefore, the original strand cannot completely be replicated and the chromosome becomes shorter every cell division, which endangers the important, informative sequence of the chromosome (figure 1)[(5-7)].

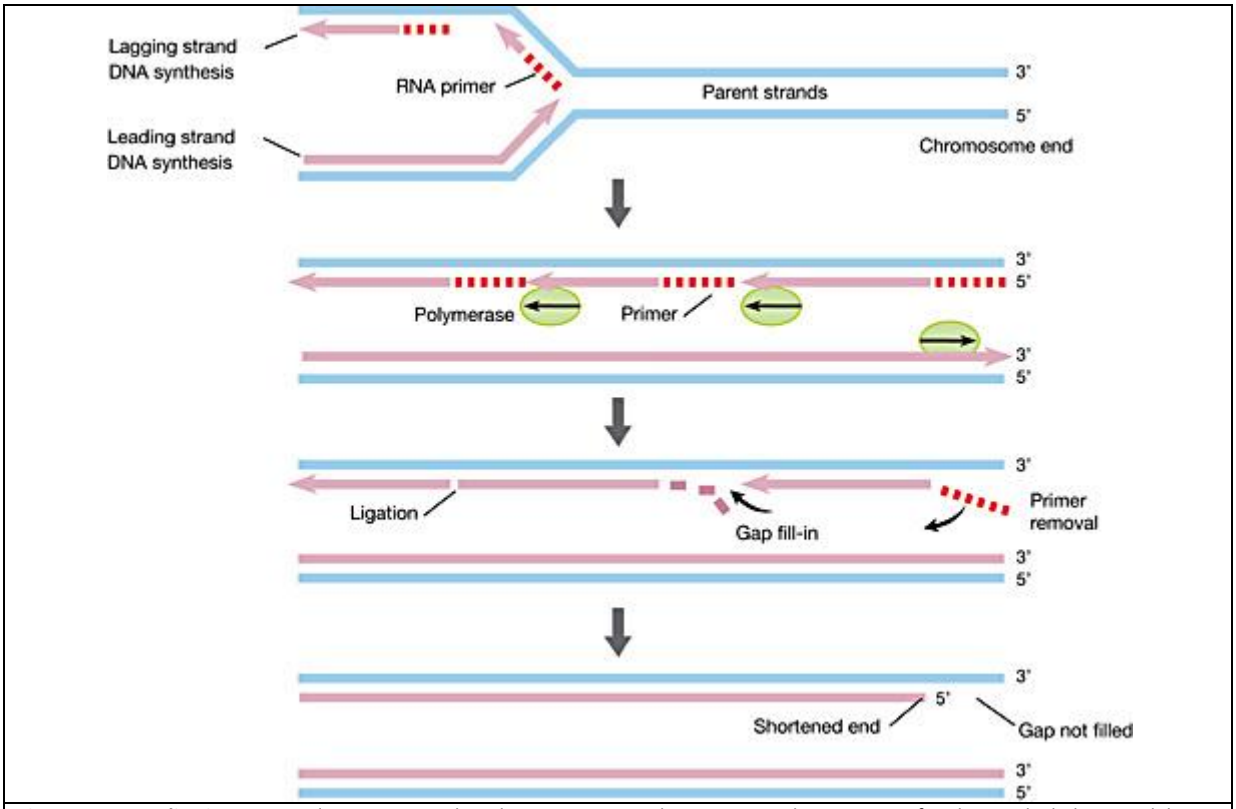


Figure 1 DNA replication. DNA replication starts when the two DNA strands are separated at an origin of replication by helicase. While separating the strands, RNA primers (red) bind to the template strand (blue) and make the starting point for DNA polymerase (green) to replicate the template strand. However, DNA polymerase can only form a new strand in a 5' to a 3' prime direction. Therefore, the strand that is opened to its 3' end needs a new RNA primer every couple of free base pairs. This occurs until the template strand is completely synthesized complementary. Hereafter, the RNA primers are removed and the arising gaps are closed by DNA polymerase. The newly synthesized strand is completely complementary on the new 3' end. However, due to the removal of the RNA primers and the absence of a 3' hydroxyl group to bind to, the new 5' end cannot be completely synthesized. This results into a new strand that is slightly shorter than the original strand[9].

At first, this shortening of telomeres is not undesirable, as proteins binding to the telomere and the specific folding of the telomeric DNA prevents the DNA from being recognized by the DNA damage machinery as double strand break. However, at a certain point, cells have proliferated so many times (in budding yeast this occurs after 60-80 generations) that the telomeres become critically short. At this point, the telomeres are not able anymore to properly fold and not all the needed proteins can bind to the telomeric DNA. Therefore, the telomeres are not properly protected anymore and are recognized as damage. This leads to a DNA damage response and the cell dies or goes into senescence. In the latter state, the cells stop growing and dividing to prevent damage to the important information containing part of the DNA. This process protects the organism against uncontrolled cell divisions, which are associated with cancer, but they also prevent the renewal and repair of tissue and therefore contribute to aging and age-related diseases [10-12].

Telomere elongation in cancer cells

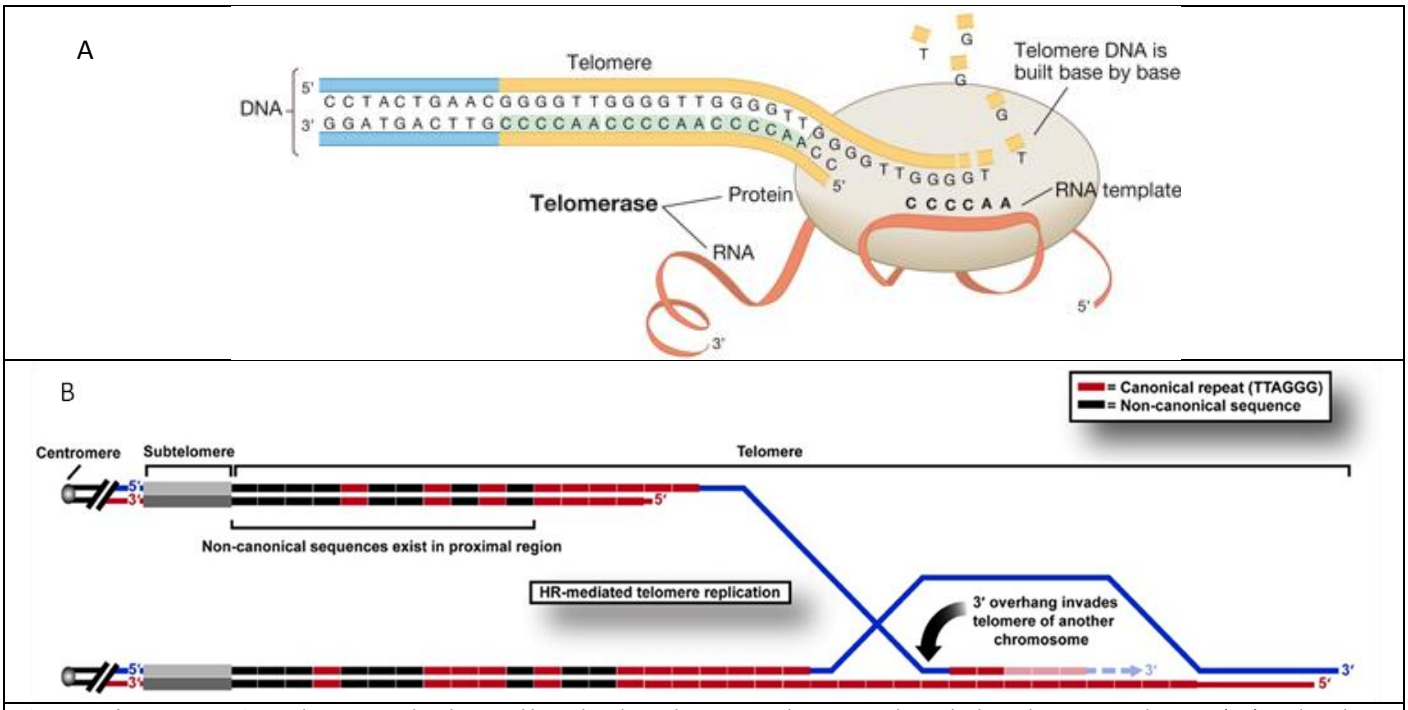
Cancer cells avoid this apoptosis and senescence by critically short telomeres, by the activation of telomere restoring mechanisms. Estimated is that 85% of the cancer cells prevent telomere shortening by reactivation of telomerase and 15% use a recombination-like mechanism called alternative lengthening of telomeres or ALT[7, 13, 14].

Telomerase is a reverse transcriptase, an enzyme that generates complementary DNA from RNA. The enzyme consists of a catalytic subunit and a RNA subunit which contains a template that is complementary to the 3' overhang sequence (in budding yeast this template sequence is ACACACACCCACACCAC (3' to 5')[4]). This RNA subunit binds to the 3' overhang and elongates this strand by addition of the $G_{2\text{-}3}(TG)_{1\text{-}6}T$ repeats. This addition of new nucleotides to the leading strand makes it possible for RNA primers to rebind to this strand and synthesize the complementary strand, just like during normal replication (figure 2A) [5-7].

Alternative lengthening of telomeres is a mechanism where cells lacking telomerase can elongate their telomeres, this occasionally induces unlimitedly proliferation. It is thought that this occurs due to a recombination-mediated mechanism, which is based on the idea that a short telomere invades into another telomere and uses this telomere as a template to elongate its own strand. Hereafter, the enlarged 3' overhang can be complementary synthesized, (figure 2B)[5, 7, 15].

Telomere elongation in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is an organism that naturally expresses telomerase. When the telomerase is inactivated, they start to senesce similar to most somatic cells. This senescing phenotype can be obtained by the inactivation of one of the subunits of telomerase; the catalytic subunit EST2, the RNA subunit TLC1, and additional subunits EST1 and EST3. The subset of the population that can overcome senescence by a telomerase-independent mechanism (ALT) are called survivors. The survivor population can be divided into two groups depending on their elongated sequences, distinguishable by southern blot and their gene requirement. This is due to that yeast telomeres consist next to the G-rich repeats, also of an X element and an optional Y' element repeat (figure 3A,B). X repeats and Y' repeats are telomere associated sequences and located in the subtelomere.


A
Telomere
DNA
5'
3'
C C T A C T G A A C G G G G T T G G G G T T G G G G T T

During survivor formation, the quantity of one X element on every chromosome does not alter. However, there are survivors that compensate their telomere loss by amplification of the Y' element, associating the Y' element repeats with the formation of survivors[18, 19]. This type of survivors are called type I survivors who have next to this amplification, also short TG_{1-3} repeats and they grow poorly but appear more frequent than type II survivors (figure 3C). The formation of these survivors depends on the presence of Pol32, Rad51, Rad52, Rad54, Rad55 and Rad57. Type II survivors require the MRX complex (Mre11, Rad50, Xrs2), Pol32, Rad52, Rad59 and Sgs1 for their formation. Further, this second type of survivors does not change their number of Y' elements but have a long heterogeneous amplification of TG_{1-3} repeats and have a similar growth rate as telomerase positive wild type cells (figure 3D,E). This second survivor type resembles the majority of human cancer cells that lengthen their telomeres by ALT [5, 13, 18, 20].

Research question

Nevertheless, the complete mechanism of how telomerase negative yeast and human cancer cells trigger themselves to start using ALT to prevent senescence or death due to critically short telomeres is not known yet. Therefore, in this study we aim to find an answer to the question: does the proteome[1] of pre-senescence cells (cells with wild-type length telomeres that have not yet been through senescence) differ from the proteome of cells with critically short telomeres and from survivor cells?

SILAC

To investigate this, we use a proteome quantification method called stable isotope labeling by amino acids in cell culture (SILAC) (figure 4). This is a method where lysine and/or arginine with a slightly higher molecular weight, are incorporated into the proteins of an organism by the use of other carbon and nitrogen isotopes ($^{13}C/^{15}N$ instead of the naturally higher abundant $^{12}C/^{14}N$). With the use of different combinations of the heavier and lighter lysine and arginine, the proteome of organisms in different conditions can be measured at the same time. To investigate the differences, the samples are pooled together early in the process to reduces the variations between the samples that might occur during preparation for mass spectrometry. Mass spectrometers recognize the small differences in size and thus the proteome of cells or organisms in different conditions or at different time points can be relatively quantified and compared [21-23].

[1] All the proteins expressed in a cell/tissue/organism at a certain time point.

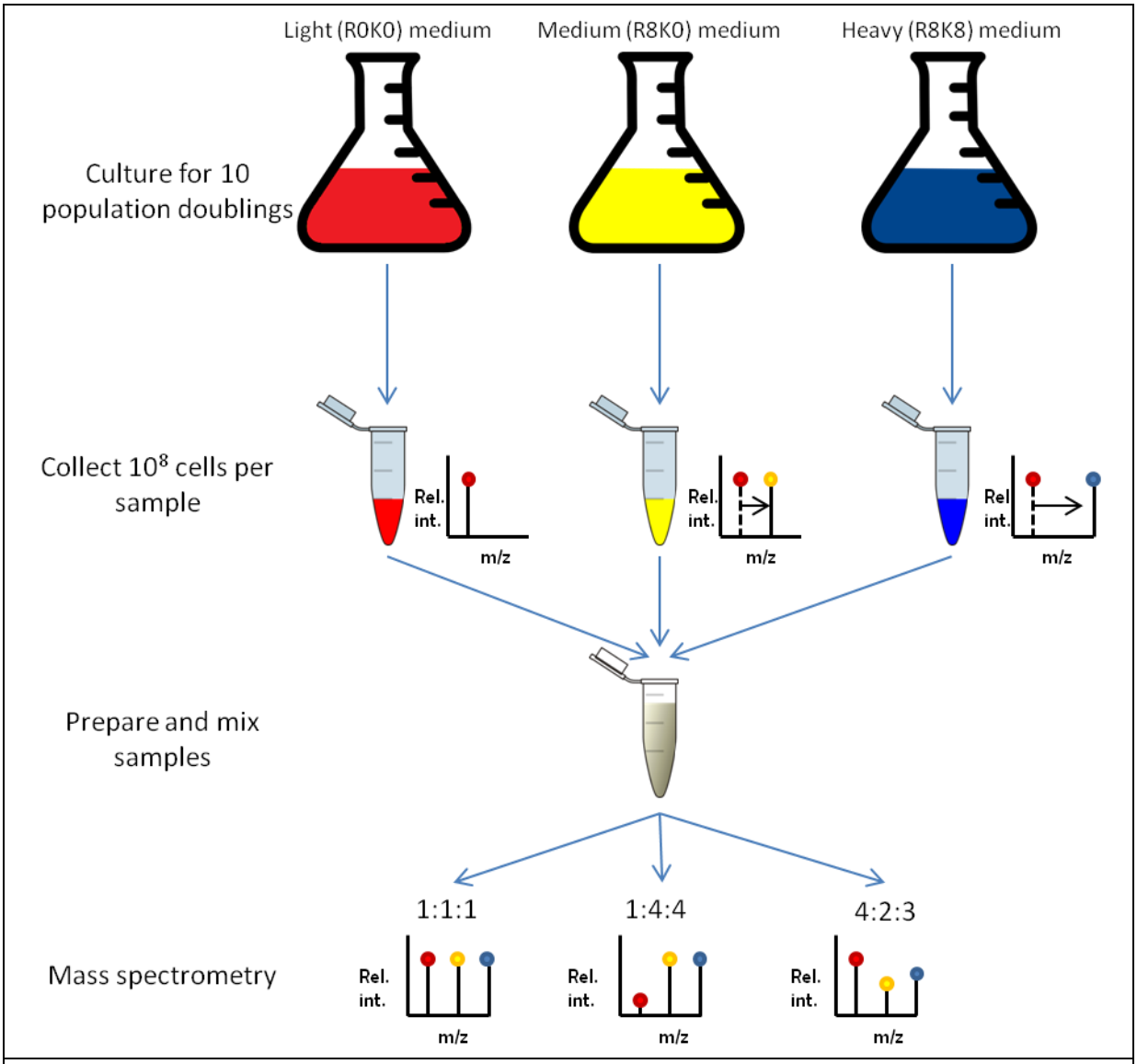


Figure 4 SILAC. At different time points (e.g. pre-senesced, senescing and survivors), budding yeast is grown for 10 population doublings in different growth media. These growth media contain normal lysine and arginine, one normal amino acid and one heavier-labeled amino acid or the heavier-labeled version of both amino acids. From all these media, an identical amount of cells is collected (10^8 cells), combined to one sample and analyzed with mass spectrometry. This provides information about the abundance of all the proteins and possible alterations in abundance between the different time points.

Chromatin remodeling genes

Even though less is known about ALT cells, a proper chromatin configuration of telomeres is thought to be essential. Since the deletion or a point mutation in some of the chromatin remodeling genes are shown to be associated with the formation of the ALT phenotype. For example, the knock down of the genes *asf1a* and *asf1b* in human lung fibroblasts and HeLa cells is shown to result in more APB formation, elevated t-SCEs and accumulation of ECTRs[14]. These are phenotypes which are associated with ALT positive cells. APBs, which stands for ALT-associated promyelocytic leukemia (PML) nuclear bodies (NBs), are PML proteins that co-localize with telomeres, telomere binding proteins and factors that are also essential for survivor formation (Rad51 and Rad52) in ALT positive cells. While in ALT negative cells, PML NBs are part of the nuclear matrix [24, 25]. The phenotype of elevation of t-SCEs, telomeric sister chromatid exchanges, is due to the recombination-like mechanism, whereby the telomeres are elongated by utilizing a sister chromatid as the template. Hence, as survivors form because of a recombination-mediated mechanism, the amount of t-SCEs will be elevated. At last ECTRs, extrachromosomal telomeric repeats, are linear, double-stranded circular (T-circles) and single-stranded circular telomeric DNA (C-circles) which are not located in the chromosome. Especially C-circles are associated with the ALT phenotype. This is because there appear to be a 750-fold more C-circles in ALT positive cells than in ALT negative cells[15].

This leads to the idea that the deletion of a chromatin remodeler gene could alters the chromatin configuration at telomeres. Which might influence the senescence or survivor formation of telomerase negative yeast. In order to study the role of chromatin remodelers genes, we cherry picked six chromatin remodelers genes: *ASF1*, *SWI3*, *SNF5*, *SET2*, *SIR2* and *IES4*, and investigated their role in senescence and survivor formation.

ASF1

ASF1 (Anti-Silencing Factor 1) is a histone chaperone that is involved in histone deposition and exchange during the assembly and disassembly of the nucleosome. This involvement in histone positioning can be replication dependent and independent[14, 26].

SWI3 & SNF5

SWI3 and SNF5 are subunits of the SWI/SNF complex (SWItch/Sucrose NonFermentable). Both SWI3 and SNF5 are involved in transcriptional activation. The complete complex is involved in the remodeling of nucleosomes and the initiation an elongation of transcription in an ATP dependent manner. Also, associations have been found between this complex and the formation of tumors[27-29].

SET2

SET2 (SET domain-containing protein 2) is a histon methyltransferase that is involved in the transcriptional repression and elongation[30, 31].

SIR2

SIR2 (Silent Information Regulator 2) encodes for a NAD+ dependent histone deacetylase that facilitates chromatin silencing in inter alia, telomeres. With this the gene expression is involved in regulating recombination, telomeric silencing, genomic stability and aging. Absence of the encoded protein can lead to loss of transcriptional silencing, decreased chromosome stability and decreased lifespan in budding yeast [32, 33].

IES4

IES4 (Ino Eighty Subunit 4) is part of the INO80 complex. Less is known about the subunit but it is proposed that Ies4 is involved in the DNA damage signaling pathway. The INO80 complex is involved in transcription, replication, cell division and DNA repair. Further, it is shown that it remodels the chromatin by moving the nucleosomes along the DNA[34-36].

Research question

We aim to investigate the role of chromatin remodeler genes during senescence and survivor formation in budding yeast. Therefore, we aim to answer the question: does the deletion of the chromatin remodeling genes *ASF1*, *SWI3*, *SNF5*, *SET2*, *SIR2* or *IES4* influence the senescence rate, survivor formation rate or survivor formation type in telomerase negative *Saccharomyces cerevisiae*?

Materials and methods

Strains

SILAC strain

Multiple strains are made to form a diploid that can generate the haploid strains W303 *est2Δ lys2Δ arg4Δ* (VI, XI and XV) strain and W303 *EST2 lys2Δ arg4Δ* (VII, XII and XVI), the process is also shown in a pedigree in figure 5 and the full genotypes of all the possible strains are shown in table 1. Initially, the parental strains W9100-2D (Ia) and W9100-17D (Ib) are transformed with an *arg4* deletion (II) and crossed with an *est2* deletion containing haploid (IV) derived from the parental diploid strain LSY1092 (III). This generates a diploid strain (V) which is heterozygous for all three interested genes and is therefore sporulated and dissected to obtain the wanted haploid strains (VI and VII). The latter haploid strain (VII) in both mating types are transformed with the wild-type *CAN1* (VIII) originated from BY4742 and crossed with each other (IX). This diploid is transformed with the est2Δ deletion, generating a diploid (X) which is sporulated and dissected to obtain the wanted haploid strains (XI and XII). Also, the parental strains W9100-2D (Ia) and W9100-17D (Ib) are transformed with an *est2* deletion (XIII) and crossed with a haploid strain containing the *lys2Δ* and *arg4Δ* deletion and the wild-type *CAN1* gene(XIV). This diploid is sporulated and dissected to obtain the wanted haploid strains (XV and XVI).

The KanMX cassette is switched into a NatMX cassette by the transformation of approximately 4 µg of EcoRI-digested pBL1 (a plasmid containing NatMX resistance gene and provided by Brian Luke, Boone Lab, University of Toronto) into a KanMX cassette containing strain.

The *can1-100* mutation is repaired by the amplification of this sequence in a wildtype BY4742 strain and this construct is transformed into the recipient strain.

Chromatin remodeling genes strains

The W303 strains *EST2/est2ΔURA3 ASF1/asf1ΔKanMX*, *EST2/est2ΔURA3 SNF5/snf5ΔKanMX*, *EST2/est2ΔURA3 SWI3/swi3ΔKanMX*, *EST2/est2ΔURA3 SET2/set2ΔKanMX*, *EST2/est2ΔURA3 SIR2/sir2ΔKanMX* and *EST2/est2ΔURA3 IES4/ies4ΔKanMX* are made by transforming a construct, where the gene is replaced by KanMX, into a W303 *EST2/est2ΔURA3* (*EST2/est2ΔURA3 ade2-1/ade2-1 leu2-3,112/leu2-3,112 his3-11/his3-11 trp1-1/trp1-1 ura3-1/ura3-1 RAD5*) strain by LiAc transformation.

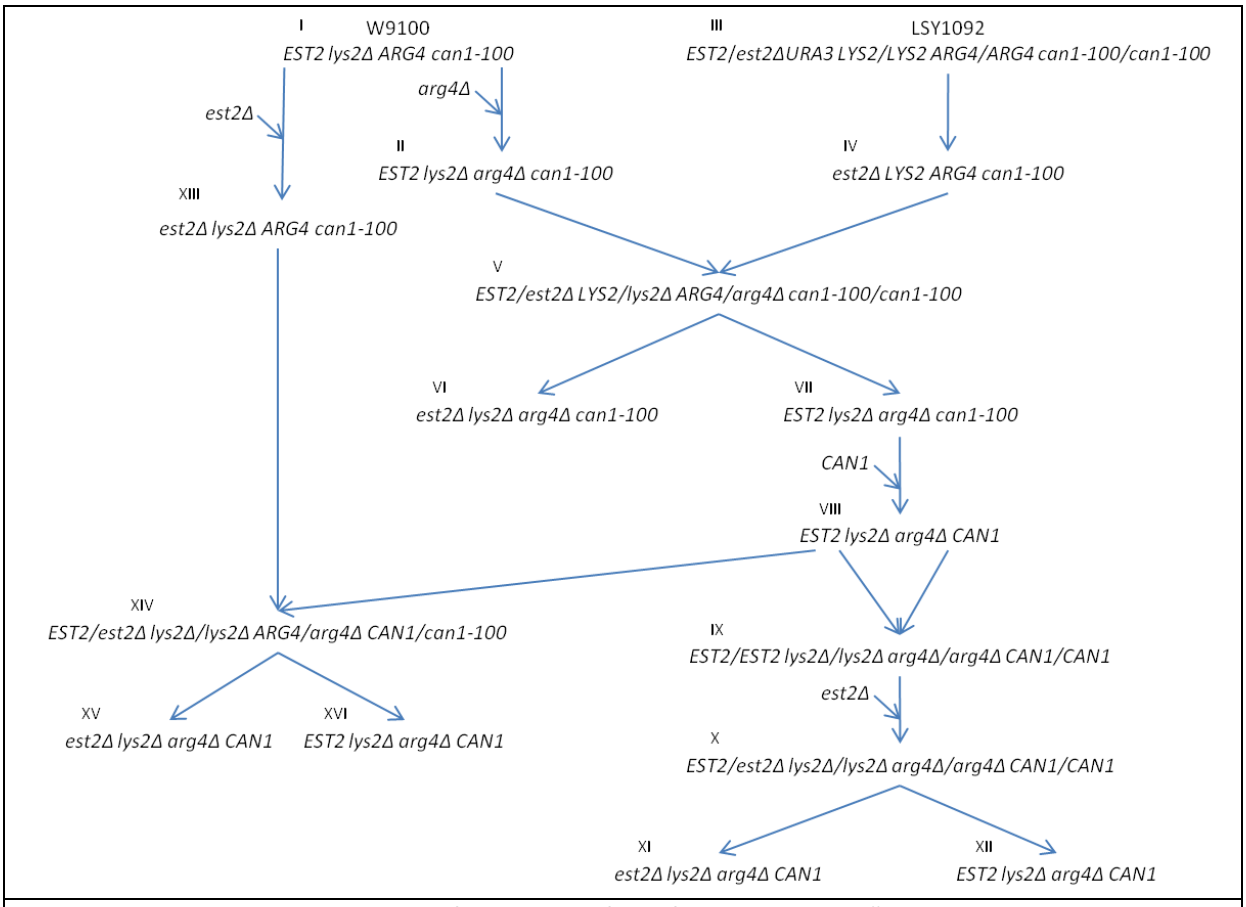


Figure 5. Pedigree of the formation of the *est2Δ lys2Δ arg4Δ* and *EST2 lys2Δ arg4Δ* strain. All the daughter strains are made with W9100-2D as *MAT α* parent, W9100-17D as *MAT a* parent or LSY1092 as *MAT α* or *Mat a* parent. Also, all the acquired haploid strains are present in both mating types. Further, the strains are made with an *est2ΔURA3* replacement as well as with an *est2ΔKanMX* replacement and with an *arg4ΔKanMX* replacement as well as with an *arg4ΔNatMX* replacement.

Table 1. The name, mating type, genotype and parental strain of all strains that should be formed during the strain formation process. [part 1/2]

Strain name (report)		Strain name (database)	Mating type	Genotype	Parental strain
I	a	W9100-2D	α	*lys2Δ ADE2 leu2-3,112 his3-11,15 ura3-1 TRP1 lys2Δ RAD5 can1-100*	Database
	b	W9100-17D	a	*lys2Δ ADE2 leu2-3,112 his3-11,15 ura3-1 TRP1 lys2Δ RAD5 can1-100*	Database
II	a	MBY1	α	*lys2Δ arg4ΔKanMX ADE2 leu2-3,112 his3-11,15 ura3-1 TRP1 RAD5 can1-100*	W9100-2D [1a]
	b	MBY2	a	*lys2Δ arg4ΔKanMX ADE2 leu2-3,112 his3-11,15 ura3-1 TRP1 RAD5 can1-100*	W9100-17D [1b]
	c	MBY3	α	*lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 TRP1 RAD5 can1-100*	W9100-2D [1a]
	d	MBY4	a	*lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 TRP1 RAD5 can1-100*	W9100-17D [1b]
III		LSY1092	α/a	*EST2/est2ΔURA3 LYS2/LYS2 ARG4/ARG4 can1-100/can1-100*	Database
IV	a	n/a	α	*est2ΔURA3 LYS2 ARG4 can1-100*	LSY1092 [III]
	b	n/a	a	*est2ΔURA3 LYS2 ARG4 can1-100*	LSY1092 [III]
V	a	n/a	α/a	*EST2/est2ΔURA3 LYS2/lys2Δ ARG4/arg4ΔKanMX ADE2/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ura3-1/ura3-1 TRP1/trp1-1 RAD5/RAD5 can1-100/can1-100*	MBY1/2 [IIa/b] + [IVa/b]
	b	n/a	α/a	*EST2/est2ΔURA3 LYS2/lys2Δ ARG4/arg4ΔNatMX ADE2/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ura3-1/ura3-1 TRP1/trp1-1 RAD5/RAD5 can1-100/can1-100*	MBY3/4 [IIc/d] + [IVa/b]

Growth media

Liquid growth media

Homemade minimal SD liquid medium without arginine and lysine is made by 1.3 g drop-out mix, 6.7 g yeast nitrogen base without amino acids, half a pellet NaOH 40 mL 50% glucose (Sigma) and 960 mL dH_2O. The drop-out mix consists of: 2.5 g adenine, 6.0 g L-aspartic acid, 6.0 g L-glutamic acid, 1.2 g L-histidine, 3.6 g L-leucine, 1.2 g L-methionine, 3.0 g L-phenylalanine, 22.5 g L-serine, 12.0 g L-threonine, 2.4 g L-tryptophan, 1.8 g L-tyrosine, 9.0 g L-valine and 1.2 g uracil (Acros Organics). Heavier labeled arginine and lysine are added to the individual 50 mL cultures as 250 µL of 82.759 mg/mL arginine and 250 µL of 124.138 mg/mL lysine.

YPD medium is made with 10 g yeast extract (Oxoid), 20 g peptone, 40 mL 50% glucose (Sigma) and 960 mL dH_2O.

Agar plates

Agar plates are made by the same recipe as liquid growth media, with the exception that for every 1 L media, 20 g agar (Invitrogen) is added. SD plates contain all the 15 amino acids mentioned above, except when mentioned otherwise. For example, SD-arginine consists of all the mentioned amino acids except arginine.

The antibiotic G418 (geneticin, Jena Bioscience) is added to the plates as 200 mg/L per plate. The antibiotic NAT (cloNAT, nourseothricin, Jena Bioscience) is added to the plates as 100 mg/L per plate.

Minimal sporulation plates are made with 10 g potassium acetate (Sigma-Aldrich), 1 g yeast extract (Oxoid), 0.5 g glucose (Sigma), 0.1 g drop-out mix, 20 g agar, 1 L dH_2O and G418. The drop-out mix consists of 2 g histidine, 10 g leucine, 2 g lysine and 2 g uracil (Sigma).

Plates that are used in this study are SD complete, SD-arginine, SD-lysine, SD-uracil, SDmin, YPD, YPD+G418, YPD+NAT, YPD+G418+NAT, SPOmin.

Strain formation

PCR for transformation

DNA needed for transformation is retrieved by amplifying the desired construct with PCR (polymerase chain reaction). PCR is done by standard protocol[37] and used primers are listed in table 2. DNA amplication is check on 1% agarose gels (MP agarose, Roche; 10x TBE buffer, Gibco Life Technologies) before transformation.

Table 1. The name, mating type, genotype and parental strain of all strains that should be formed during the strain formation process. [part 2/2]

Strain name (report)		Strain name (database)	Mating type	Genotype	Parental strain
VI	a	n/a	α	*est2ΔURA3 lys2Δ arg4ΔKanMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 can1-100*	[V]
	b	n/a	a	*est2ΔURA3 lys2Δ arg4ΔKanMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 can1-100*	[V]
	c	n/a	α	*est2ΔURA3 lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 can1-100*	[V]
	d	n/a	a	*est2ΔURA3 lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 can1-100*	[V]
VII	a	n/a	α	*EST2 lys2Δ arg4ΔKanMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 can1-100*	[V]
	b	n/a	a	*EST2 lys2Δ arg4ΔKanMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 can1-100*	[V]
	c	n/a	α	*EST2 lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 can1-100*	[V]
	d	n/a	a	*EST2 lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 can1-100*	[V]
VIII	a	MBY9	α	*EST2 lys2Δ arg4ΔKanMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[VII]
	b	MBY8	a	*EST2 lys2Δ arg4ΔKanMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[VII]
	c	MBY7	α	*EST2 lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[VII]
	d	MBY6	a	*EST2 lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[VII]
IX	a	n/a	α/a	*EST2/EST2 lys2Δ/lys2Δ arg4ΔKanMX/arg4ΔKanMX ADE2/ADE2 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ura3-1/ura3-1 RAD5/RAD5 CAN1/CAN1*	[VIIIa] +[VIIIb]
	b	n/a	α/a	*EST2/EST2 lys2Δ/lys2Δ arg4ΔNatMX/arg4ΔNatMX ADE2/ADE2 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ura3-1/ura3-1 RAD5/RAD5 CAN1/CAN1*	[VIIIa] +[VIIIb]
X	a	n/a	α/a	*EST2/est2ΔURA3 lys2Δ/lys2Δ arg4ΔKanMX/arg4ΔKanMX ADE2/ADE2 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ura3-1/ura3-1 RAD5/RAD5 CAN1/CAN1*	[IXa]
	b	n/a	α/a	*EST2/ est2ΔURA3 lys2Δ/lys2Δ arg4ΔNatMX/arg4ΔNatMX ADE2/ADE2 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ura3-1/ura3-1 RAD5/RAD5 CAN1/CAN1*	[IXb]
	c	n/a	α/a	*EST2/ est2ΔKanMX lys2Δ/lys2Δ arg4ΔNatMX/arg4ΔNatMX ADE2/ADE2 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ura3-1/ura3-1 RAD5/RAD5 CAN1/CAN1*	[IXc]
XI	a	n/a	α	*est2ΔURA3 lys2Δ arg4ΔKanMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[Xa]
	b	n/a	a	*est2ΔURA3 lys2Δ arg4ΔKanMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[Xa]
	c	n/a	α	*est2ΔURA3 lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[Xb]
	d	n/a	a	*est2ΔURA3 lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[Xb]
	e	n/a	α	*est2ΔKanMX lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[Xc]
	f	n/a	a	*est2ΔKanMX lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[Xc]
XII	a	n/a	α	*EST2 lys2Δ arg4ΔKanMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[Xa]
	b	n/a	a	*EST2 lys2Δ arg4ΔKanMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[Xa]
	c	n/a	α	*EST2 lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[Xb] or [Xc]
	d	n/a	a	*EST2 lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[Xb] or [Xc]
XIII	a	n/a	α	*est2ΔURA3 lys2Δ ARG4 ADE2 leu2-3,112 his3-11,15 ura3-1 TRP1 lys2Δ RAD5 can1-100*	W9100-2D [Ia]
	b	n/a	a	*est2ΔURA3 lys2Δ ARG4 ADE2 leu2-3,112 his3-11,15 ura3-1 TRP1 lys2Δ RAD5 can1-100*	W9100-17D [Ib]
	c	n/a	α	*est2ΔKanMX lys2Δ ARG4 ADE2 leu2-3,112 his3-11,15 ura3-1 TRP1 lys2Δ RAD5 can1-100*	W9100-2D [Ia]
	d	n/a	a	*est2ΔKanMX lys2Δ ARG4 ADE2 leu2-3,112 his3-11,15 ura3-1 TRP1 lys2Δ RAD5 can1-100*	W9100-17D [Ib]
XIV		MBY5	α/a	EST2/est2ΔKanMX lys2Δ/lys2Δ ARG4/arg4ΔNatMX ADE2/ADE2 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 ura3-1/ura3-1 TRP1/TRP1 RAD5/RAD5 CAN1/can1-100	MBY7 [VIIIc] + [XIIId]
XV	a	n/a	α	*est2ΔKanMX lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[XIV]
	b	n/a	a	*est2ΔKanMX lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[XIV]
XVI	a	n/a	α	*EST2 lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[XIV]
	b	n/a	a	*EST2 lys2Δ arg4ΔNatMX ADE2 leu2-3,112 his3-11,15 ura3-1 RAD5 CAN1*	[XIV]

Transformation

Transformations are done by the LiAc (lithium acetate) method. For this, the recipient strain is grown overnight while rotating (56 rpm) in 5 mL YPD at 30 °C. In the morning, this culture is re-diluted to an OD_{600} of 0.1 in 20 mL YPD and grown while shaking (200 rpm) at 30 °C to mid-log phase culture. At this point, the cells are pelleted (3K rpm for 5 minutes) and washed with 1 mL dH_2O. Next, the cells are washed with 0.5 mL 1xTE/LiAc (10x TE: 100 mM Tris-Cl (pH 7.4), and 10mM EDTA; 10x LiAc: 1 M lithium acetate) and resuspended in 150 µL 1xTE/LiAc. Per transformation, 50 µL of this cell suspension, 15 µL PCR product, 5 µL of 10 mg/mL salmon sperm carrier DNA (Sigma) and 400 µL 40% PEG4000 solution is mixed and incubated while rotating (56 rpm) for 30 minutes at 30 °C. Hereafter, the cells are heat shocked at 42 °C for 15 minutes, collected by centrifugation (2.5K rpm for 1 minute) and incubated in 200 µL YPD while rotating at 30 °C for 2-4 hours. Last, the cells are resuspended in 100 µL dH_2O and plated on appropriate selective media and grown for 2 days at 30°C.

Gene replacement confirmation

The strains are check for deletion of the genes, by replacement by different cassettes, by the growth on selective media plates. The used selective media plates are shown in table 3. Also, the strains are checked by PCR. For this, genomic DNA is isolated with the Wizard genomic DNA purification kit (Promega) and is amplified with the PCR mix, PCR protocol (annealing temperature 58 °C) and primers mentioned above ("PCR for transformation" and table 2). Additionally, KanMX replacements are checked by PCR with a primer (KanB[38]) that binds to a sequence that is only present in the KanMX cassette. Used primers are shown in table 4.

Liquid senescence assay

SILAC strain (*est2Δ arg4Δ lys2Δ*)

The EST2 arg4Δ lys2Δ strain is inoculated in 2 mL SD complete(Sigma-Aldrich) and the cultured at 30 °C while rotating (56 rpm) for 24 hours. Hereafter, the OD_{600} is measured in order to get the cell density of the culture and the culture is diluted to $2.5 \cdot 10^4$ cell/mL in 50 mL of complete media lacking arginine and lysine (SD –arginine – lysine) and supplemented with labeled arginine and lysine. Cells were grown at 30 °C at 200 rpm for 24 hours. From this culture the cell morphology of at least 300 cells is checked and counted by light microscopy (40x objective), $1.4 \cdot 10^7$ cells are collected and stored at -20 °C for southern blot, $1 \cdot 10^8$ cells are collected, washed with MilliQ, snap frozen with liquid nitrogen and stored in -80 °C for SILAC.

The est2Δ arg4Δ lys2Δ strain is inoculated in 2 mL SD complete and the cultured at 30 °C while rotating (56 rpm) for 24 hours. Next, the OD_{600} is measured and the culture is diluted to $2 \cdot 10^5$ cell/mL in 100 mL SD complete and grown at 30 °C while rotating (200 rpm) for 24 hours. Dilution of the culture and measurement are repeated evey 24h for 8 day. Aliquots are taken every day for microscopy, southern blot and SILAC as described above. When culture reaches normal growth, indicative as appearance of survivors, 200 cells are plated on a YPD plate and grown for 2 days at 30 °C. Grown colonies are inoculated in 2 mL SD complete and cultured at 30 °C while rotating (56 rpm) for 24 hours. At last, the OD_{600} is measured and the culture is diluted to $2.5 \cdot 10^4$ cell/mL in 50 mL SD with labeled arginine and lysine and grown at 30 °C while rotating (200 rpm) for 24 hours. Also for this culture southern blot, microscopy and SILAC samples are acquired.

Table 2. PCR primers for amplification of constructs for transformation.

Standard gene name	Systematic gene name	Forward primer	Reverse primer
ARG4	YHR018C	ATCTGCCAAGGCTCCATC	GTCTCATGGCCATTTGCTTC
EST2	YLR318W	AACCATAACTAACACGCCCTC	GAAGATGTGGGAGATGGAAAG
CAN1	YEL063C	AGAGTGGTTGCGAACAGAGT	TGATATAAGAGCGCCCACTGG
ASF1	YJL115W	GATGGTAATGCCTTGGCGA	TAGGGCGTGTGGCGTAGT
SWI3	YJL176C	ACCGCCGTGGTTACGATG	GAGAAGCCAAGTCAAGTGACG
SNF5	YBR289W	GTCCACAGGTGCTTGAAGG	GTCTCTAGTTCGTCCTGCG
SET2	YJL168C	CCGCTTAGAATACCTCACAC	CGACGCTGACCCTTTATATG
SIR2	YDL042C	CATCTAGCACTCCTTCCAACC	GCTATACCACCACCTCCTTTC
IES4	YOR189W	CAGGGAAACAAACTGCATGG	AATCGTCGCCATTCCTGTC

Table 3. Selective medias on which the absence of genes or the presence of cassettes is tested.

Selective media	Deletion/replacement	Growth on selective media due to deletion/replacement
SD without arginine	*arg4Δcassette*	No growth
SD without lysine	*lys2Δcassette*	No growth
SD complete	*CAN1*	Growth
YPD with G418*	*geneΔKanMX*	Growth
YPD with NAT**	*geneΔNatMX*	Growth
SD without uracil	*geneΔURA3*	Growth

* G418: geneticin, KanMX has geneticin resistance.
** NAT: nourseothricin, NatMX has nourseothricin resistance.

Table 4. PCR primers for confirmation of KanMX insertion.

Standard gene name	Systematic gene name	Forward primer
ARG4	YHR018C	TCGGCGTCCCAATCTTTTT
EST2	YLR318W	CGTTCCAACCCAAATACTCTT
ASF1	YJL115W	TCAACAACCGGGCGACTAGG
SWI3	YJL176C	TGGCAAGTACGGCCACACAG
SNF5	YBR289W	TTCACCCGCTTCGACGCAAC
SET2	YJL168C	AGCTTACCGCCTGGAGTGTTG
SIR2	YDL042C	AGAGATTTGGCACCACGAGC
IES4	YOR189W	GACCTCCACCTATGCGAGCAC

Growth conditions

The strains are individually inoculated in 2 mL YPD and cultured at 30 °C while rotating (56 rpm) for 24 hours. Next, the OD_{600} is measured and culture is diluted to $2 \cdot 10^5$ cell/mL in 5 mL YPD and grown at 30 °C while rotating (200 rpm) for 24 hours. Hereafter, for 8 days, every 24 hours the OD_{600} is measured, the cells are re-diluted to $2 \cdot 10^5$ cell/mL in 5 mL YPD and $1.4 \cdot 10^7$ cells are collected and stored at -20 °C for southern blot.

Senescence assay on plate

The chromatin remodeling strains are also streaked for single colonies on YPD plates. Hereafter, they are grown for 48 hours, (part of) a single colony is picked and is restreaked on a new YPD plate. This is repeated 7 times and scored based upon Rizki's and Lundblad's scoring system[39]. Who scored the severeness of senescence of the strain based upon the number of colonies and the colony size.

Southern blot

Genomic DNA is extracted with the Wizard genomic DNA purification kit from Promega. Overnight, 37.5 µL genomic DNA is digested with 2.5 µL XhoI (New England Biolabs) and 5 µL Neb 4 buffer (New England Biolabs) at 37 °C. Of the digested DNA, 10 µg is loaded on a 1% agarose gel at 110V. The gel is incubated in 0.25N HCL for 15 minutes for depurination and 30 minutes in 0.4N NaOH for denaturation. Next, the DNA is transferred to a positively charged nylon membrane by the vacuum transfer method (5 Hg for 90 minutes in 10xSSC, pH 7.0 (87.65g NaCl, and 44.1g sodiumcitrate, in 1 L dH_2O)). Hereafter, the membrane is rocked for 1 hour at room temperature in denaturation solution (1.5M NaCl and 0.5M NaOH) and twice for 10 minutes in neutralization solution, pH 7.2 (0.5M Tris-HCl, Serva, and 1M NaCl). Next, the membrane is pre-hybridized in 20 mL pre-warmed DIG easy hyb buffer (Roche) for 1 hour at 39 °C while rotating (10 rpm), then overnight the membrane is incubated in 5 mL TG DIG-labeled probe (labeled according to the DIG oligonucleotide 3'-end labeling KIT, 2ng generation, Roche, and diluted 1:1000 in DIG easy hyb buffer) at 39 °C while rotating. Thereafter, the membrane is washed twice with pre-warmed 2x SSC + 0.1% SDS (10x SDS) for 5 minutes at 39 °C while rotating and twice with pre-warmed 0.5x SSC + 0.1% SDS for 20 minutes at 39 °C while rotating. Next, the membrane is rinsed in 5x DIG wash buffer, pH 7.5 (58g/L maleic acid, 43.8g/L NaCl and 1.5 % Tween-20 in dH_2O) and blocked in 1x blocking solution (10x blocking solution, Roche, diluted in maleic acid buffer (11.67g/L maleic acid and 8.76g/L NaCl in dH_2O) for 30 minutes at room temperature while rotating. Then, the membrane is incubated in AP-coupled anti-DIG FAB (Roche, diluted 1:10.000 in blocking solution) for 30 minutes at room temperature while rotating. Next, the membrane is washed four times for 15 minutes at room temperature with 1x DIG wash buffer and incubated with DIG detection buffer (15.8g Tris-HCl and 5.8g NaCl in 1 L dH_2O) for 5 minutes at room temperature. Last, the membrane is incubated in 1 mL CSPD (Roche, diluted 1:100 in DIG detection buffer) in the dark for 5 minutes at room temperature and 15 minutes at 37 °C, whereafter the exposure is measured with the Biorad Chemi Doc MP Imaging System.

Results

SILAC strain

The first project is based upon the idea that the proteome of yeast changes during senescence and survivor formation. Hence, the proteome of yeast with wild-type length telomeres might be different from the proteome of yeast with critically short telomeres and/or the proteome of survivor yeast. These differences are characterized using liquid senescence assay method in combination with the SILAC method.

Strain formation

SILAC is based on the incorporation of labeled amino acid into newly synthesized proteins. The use of different labeled amino acids, single or in combination, made it possible to analyze multiple conditions at one time. In order to allow the yeast to fully incorporate the labeled amino acids, we created a strain that is deficient in the synthesis of two amino acids: arginine and lysine. The inability to synthesis these two essential amino acids itself (auxotrophic), obligates the yeast to incorporate the amino acids (heavy-labeled or light-labeled) from the media in all the newly synthesized proteins. To obtain this arginine and lysine auxotrophic strain, we made the genes *ARG4* and *LYS2* dysfunctional by replacing the genes with an antibiotics resistance gene cassette. The *ARG4* gene encodes for an argininosuccinate lyase, which catalyzes the final step of arginine biosynthesis pathway and the *LYS2* gene encodes for an alpha aminoadipate reductase, which catalyzes the fifth of seven steps of lysine synthesis pathway. Furthermore, we inactivated telomerase in this strain by knocking out *EST2*, hereby the cells will be able to senesce and form survivors. However, to perform our study on a clonal population with similar telomere lengths and no senescence before the start of the experiments, we made the arginine and lysine auxotrophic strain also heterozygous for *EST2*. Therefore, due to its one functional copy, all the cells can elongate their telomeres by telomerase until the point of dissection for haploid, auxotrophic, telomerase negative cells at the start of an experiment.

Initially, an *EST2/est2ΔURA3 ARG4/arg4ΔKanMX LYS2/lys2Δ* strain was generated to obtain an *est2ΔURA3 arg4ΔKanMX lys2Δ* strain. Dissection of this diploid and selection on appropriate selective media enables us to determine the genotype of each individual spore and acquire the strain. Based upon Mendel's laws, we were able to make a chart of the eight expected spore genotypes, listed in table 4, and the amount of times these genotypes appeared. However, we were unable to obtain the *est2ΔURA3 arg4ΔKanMX lys2Δ* strain. Two other genotypes; *EST2 arg4ΔKanMX LYS2* and *est2ΔURA3 arg4ΔKanMX LYS2* were also not able to be selected by this method. In contrast, *EST2 arg4ΔKanMX lys2Δ* appeared more often than expected.

Table 4. Distribution of genotypes in dissected tetrads; *EST2/est2ΔURA3 ARG4/arg4ΔKanMX LYS2/lys2Δ*. All eight possible genotypes and the number of times they are observed are listed. Together with the amount of isolates with this genotype that are expected to be found and an alternative expectance. The alternative expected number is based upon the idea that *arg4ΔKanMX* prevents growth on SD-lys and SD-ura. Dissected tetrads: 33. *est2ΔURA3* is selected on SD-ura, *argΔKanMX* is selected on YPD+G418 and SD-arg, *lys2Δ* is selected on SD-lys.

Genotype	n	Expected n	Alternative expected n
EST2 ARG4 LYS2	23	16,5	16,5
EST2 ARG4 lys2Δ	17	16,5	16,5
EST2 arg4ΔKanMX LYS2	0	16,5	0
EST2 arg4ΔKanMX lys2Δ	66	16,5	66
est2ΔURA3 ARG4 LYS2	9	16,5	16,5
est2ΔURA3 ARG4 lys2Δ	17	16,5	16,5
est2ΔURA3 arg4ΔKanMX LYS2	0	16,5	0
est2ΔURA3 arg4ΔKanMX lys2Δ	0	16,5	0
Total	132	132	132

All eight possible genotypes should appear in a similar ratio if the genes are not genetically linked together, but this did not occur. The genetic link between all our deleted genes was unlikely to be the cause, due to their different chromosomal position in the genome. We also observed that surprisingly *arg4Δ* mutants were growing on rich media, but not synthetic media. Strains containing *est2ΔURA3* or *LYS2* are respectively selected by growth on SD-URA and SD-lys. Hence, no growth on these media automatically suggests them to be *EST2* and *lys2Δ*, even though this might not be true. Therefore, based upon the idea that the *arg4* deletion might inhibit growth on minimal media and therefore prevent selection of *EST2* and *lys2Δ* on synthetic media, we tried to investigate this hypothesis. This prevention of growth would result in no strains with the genotype *EST2 arg4ΔKanMX LYS2*, *est2ΔURA3 arg4ΔKanMX LYS2* or *est2ΔURA3 arg4ΔKanMX lys2Δ*. All these strains will appear as *EST2 arg4ΔKanMX lys2Δ*, which is fitting with our observations.

By searching through databases and the finding that the *arg4Δ* strains are capable of growing on YPD media but not on synthetic minimal growth media, we found that *CAN1* encodes for an arginine permease and is required for the uptake of arginine from the growth media. However, originally W303 has a *can1-100* mutation in this gene. Therefore, W303 yeast is not capable of taking up arginine from the media, and its growth depends on the synthesis of arginine by the organism itself. Thus, because of the *arg4* deletion, W303 yeast cannot acquire arginine for the synthesis of new proteins and therefore the cell will not grow and replicate itself. Therefore, we restored the full version of the *CAN1* gene into our engineered strain and found that yeasts are able to grow again on minimal media.

To obtain the diploid strain, we have mated the haploid *EST2 arg4Δ lys2Δ CAN1* strain with an opposite mating type *EST2Δ arg4Δ lys2Δ CAN1* strain. However, when two *EST2 arg4Δ lys2Δ CAN1* strains are mated, no zygotes were able to form after four to six hours incubation time. These strains take approximately 24 hours to form zygotes. Hereafter, these diploids have been transformed with an *est2Δ* construct to from an *EST2/est2Δ arg4Δ/arg4Δ lys2Δ/lys2Δ CAN1/CAN1* strain. However, these zygotes (nor the untransformed diploids) are not able to sporulate within eight days on normal sporulation media, enriched sporulation media or minimal sporulation media.

(This applies to *EST2/EST2 arg4ΔKanMX/arg4ΔKanMX lys2Δ/lys2Δ CAN1/CAN1*, *EST2/EST2 arg4ΔNatMX/arg4ΔNatMX lys2Δ/lys2Δ CAN1/CAN1*, *EST2/est2ΔURA3 arg4ΔKanMX/arg4ΔKanMX lys2Δ/lys2Δ CAN1/CAN1*, *EST2/est2ΔURA3 arg4ΔNatMX/arg4ΔNatMX lys2Δ/lys2Δ CAN1/CAN1* and *EST2/est2ΔKanMX arg4ΔNatMX/arg4ΔNatMX lys2Δ/lys2Δ CAN1/CAN1*)

In contrast, the strains *EST2 arg4ΔNatMX lys2Δ CAN1* and *est2ΔKanMX ARG4 lys2Δ can1-100* mate normally to an *EST2/est2ΔKanMX ARG4/arg4ΔNatMX lys2Δ/lys2Δ CAN1/can1-100* strain. This diploid also sporulates normally, tetrads segregate in their expected ratios when divided by dissection and all possible genotypes appear in the expected ratios , table 5. For these reasons, we used this last diploid strain to obtain the haploid arginine and lysine auxotrophic, telomerase negative strain for our survivor formation analysis.

Table 5. Distribution of genotypes in dissected tetrads; *EST2/est2ΔKanMX ARG4/arg4ΔNatMX lys2Δ/lys2Δ CAN1/can1-100.* All eight possible genotypes, the number of times they appeared and the amount of isolates with this genotype that are expected to be found are listed. Dissected tetrads: 25. *est2ΔKanMX* is selected on YPD+G418, *argΔNatMX* is selected on YPD+Nat and SD-arg, *lys2Δ* is selected on SD-lys, *CAN1* is selected on SD complete.
* presence of the *can1-100* mutation can only be determined when the strain is *arg4Δ*. When the strain is *ARG4* it will grow on minimal media, independent of containing *CAN1* or *can1-100*.

Genotype	n	Expected n
EST2 ARG4 lys2Δ CAN1 *EST2 ARG4 lys2Δ can1-100 **	27	25
EST2 arg4ΔNatMX lys2Δ CAN1	13	12,5
EST2 arg4ΔNatMX lys2Δ can1-100	10	12,5
est2ΔKanMX ARG4 lys2Δ CAN1 *est2ΔKanMX ARG4 lys2Δ can1-100**	23	25
est2ΔKanMX arg4ΔNatMX lys2Δ CAN1	13	12,5
est2ΔKanMX arg4ΔNatMX lys2Δ can1-100	14	12,5
Total	100	100

Liquid senescence assay

Due to its resembles to human ALT cancer cells, we were mainly interested in type II survivor formation. This type is, because of its growth rate advantage, more abundant in liquid cultures than type I survivors (as mentioned in the introduction). Therefore, we preformed a liquid senescence assay to follow the daily change in population density, type (pre-senescence, senescence, survivor type) and proteomics. We started the assay from a clonal population to avoid differences in senescence rate and initial telomere length.

Two *est2ΔKanMX arg4ΔNatMX lys2Δ CAN1* isolates from an *EST2/est2ΔKanMX ARG4/arg4ΔNatMX lys2Δ/lys2Δ CAN1/can1-100* tetrad, have been followed through their senesces and survivor formation, figure 6. The population density of the first isolate (#1 ●) decreased with $1.4 \cdot 10^6$ cells/mL every population doubling in the first four days, leading to a critical senescence point between day 4 and 5. Hereafter, the population restores itself in approximately one day to a population density similar to the pre-senescing population density. The population from the second isolate (#2 ■) reduces $7.4 \cdot 10^5$ cells/mL every population doubling in the first four days, but the point of critical senescence and recovery of the population density to original values cannot be seen clearly. However, when looked at the compositions of the cultures, the change in morphology during the assays is similar for the first and the second strain, figure 7. Initially they have a comparable composition as telomerase positive wild type cells: high amounts of unbudded cells and cells with a large bud, and 10-20% of the cells in the population are cells with a small bud. During the period where the population doublings decreases, the amount of cells with a small bud decreases and the amount of dead cells increases, while the amount of unbudded cells and cells with a large bud stays unchanged. When the population doubling rate increases again, the amount of unbudded cells and cells with a small bud increases, the amount of dead cells and cells with a large bud in the population decreases. When single cells are picked from the recovered culture and grown to a new population, all these survivors show different morphological compositions, figure 8. However, on average, all of these populations showed a high and similar amount of unbudded cells and cells with a small bud, slightly less cells with a large bud, and nearly no dead cells.

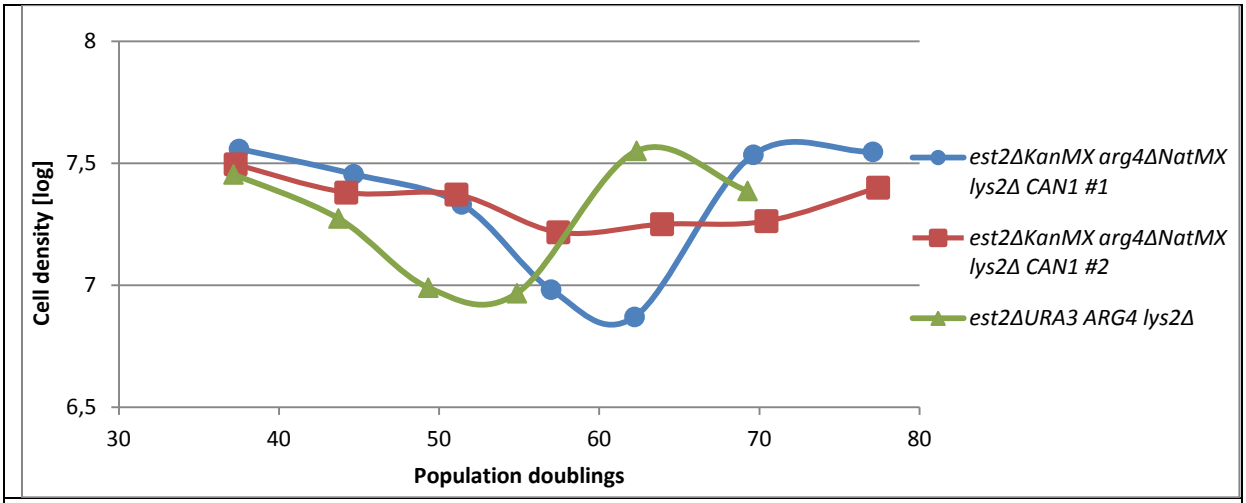
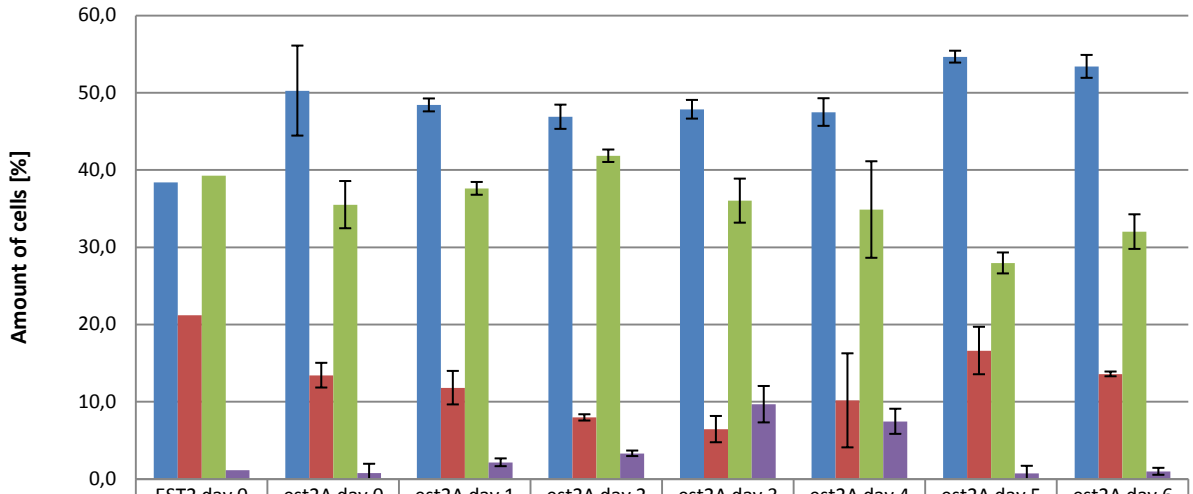
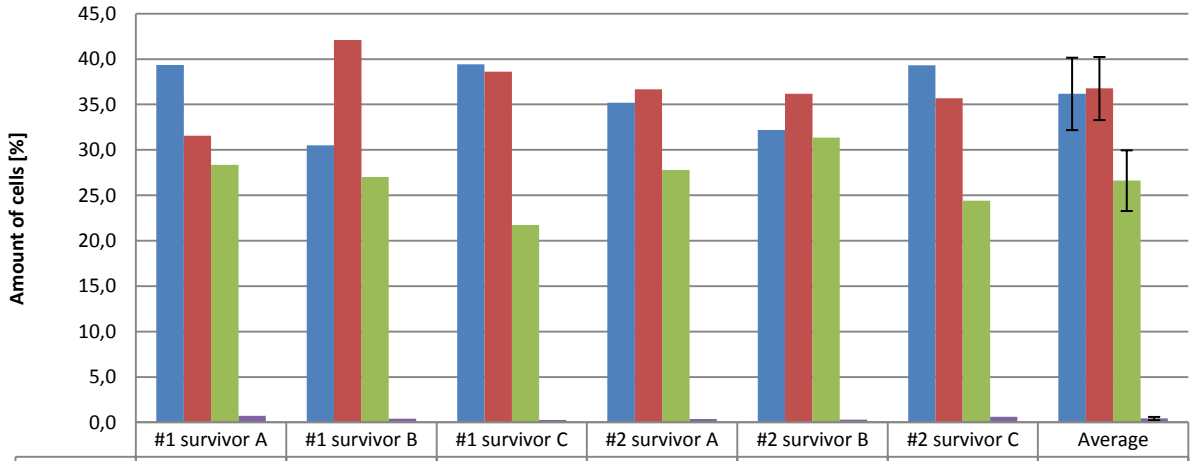


Figure 6 Liquid senescence assay curve. Two *est2ΔKanMX arg4ΔNatMX lys2Δ CAN1* isolates from different tetrads but the same diploid strain, and an *est2ΔURA3 ARG4 lys2Δ can1-100* isolate were grown and every 24 hours re-diluted to the same start density in new growth media. The population originating from the first isolate, #1, has a senescence rate of -0.028 (decrease of $1.4 \cdot 10^6$ cells/mL per population doubling) and a survivor formation rate of 0.090 (increase of $3.6 \cdot 10^6$ cells/mL per population doubling). The population originated from the second isolate, #2, has a senescence rate of -0.012 (decrease of $7.4 \cdot 10^5$ cells/mL per population doubling) and a survivor formation rate of 0.011 (increase of $5.4 \cdot 10^5$ cells/mL per population doubling) (based upon that the strain has its critical senescence point at day 4). The population originated from the third isolate, *est2ΔURA3 ARG4 lys2Δ can1-100*, has a senescence rate of -0.038 (decrease of $1.5 \cdot 10^6$ cells/mL per population doubling) and a survivor formation rate of 0.078 (increase of $3.5 \cdot 10^6$ cells/mL per population doubling).



	EST2 day 0	est2Δ day 0	est2Δ day 1	est2Δ day 2	est2Δ day 3	est2Δ day 4	est2Δ day 5	est2Δ day 6
unbudded	38,4	50,3	48,4	46,9	47,8	47,5	54,7	53,4
small bud	21,2	13,4	11,8	8,0	6,5	10,2	16,6	13,6
large bud	39,3	35,5	37,6	41,8	36,0	34,9	28,0	32,0
dead	1,1	0,8	2,2	3,3	9,7	7,5	0,8	1,0

Figure 7 Morphology of the population at every measuring time point during the senescence assay. The percentage of cells which are unbudded, have a small bud, have a large bud or are dead at day 0 in *EST2 arg4ΔNatMX lys2Δ CAN1* and of both *est2ΔKanMX arg4ΔNatMX lys2Δ CAN1* strains at day 0 to day 6.



	#1 survivor A	#1 survivor B	#1 survivor C	#2 survivor A	#2 survivor B	#2 survivor C	Average
unbudded	39,4	30,5	39,4	35,2	32,2	39,3	36,2
small bud	31,6	42,1	38,6	36,7	36,2	35,7	36,8
large bud	28,4	27,0	21,7	27,8	31,3	24,4	26,6
dead	0,7	0,4	0,3	0,4	0,3	0,6	0,4

Figure 8 Morphology of different survivor populations. The percentage of cells which are unbudded, have a small bud, have a large bud or are dead in a survivor population. All survivor populations originate from one survivor cell, which is a different survivor for every culture. From both strains, three survivors are grown to a population.

Also an *est2ΔURA3 ARG4 lys2Δ can1-100* isolate from a *EST2/est2ΔURA3 ARG4/arg4ΔKanMX lys2Δ/lys2Δ can1-100/can1-100* tetrad has been followed through its senesces and survivor formation, figure 6. The population density of this third isolate (▲) decreased with $1.5 \cdot 10^6$ cells/mL every population doubling in the first three days, leading to a critical senescence point between measuring day 3 and 4. Hereafter, the population restores itself in approximately one day to a population density comparable to the pre-senescing population density.

Southern blot

To determine if the telomeres truly shorten, at what time point survivors start to appear in the population and what type of survivors are formed, a southern blot was preformed. For this, every day during the senescence assay, a sample was collected and prepared for detection of subtelomeres and telomeres by southern blot from the third isolate, *est2ΔURA3 ARG4 lys2Δ can1-100*. The telomeres of the diploid with active telomerase (*EST2/est2ΔURA3 ARG4/arg4ΔKanMX lys2Δ/lys2Δ can1-100/can1-100*) are longer than those of the telomerase negative strain, figure 9. Comparison of figure 6 and figure 9 shows that shortening of telomeres is correlated with the decrease in population density of the telomerase negative isolate. However, when the critical senescence point is passed (after day 3), a different pattern starts to appear. This pattern is associated with elongated telomeres by ALT of a type II survivor. For all populations this pattern is identical, however, when new populations are grown from one single survivor cell, all populations have different heterogeneous patterns, which is associated with the many TG repeats of a type II survivor.

Conclusion

Via different and sometimes unsuccessful methods, we were able to engineer the *est2Δ arg4Δ lys2Δ* strain, with the restoration of the wild-type *CAN1* gene, this strain was also capable to grow and replicate on minimal media.

Further, although the three different followed isolate give different senescence curves, their morphology shows that they all senesce and restore their population in a similar manner. This suggests that even though the populations have a different rate in senescence and survivor formation, they senesce and recover by a similar process.

At last, the southern blot shows that correlated with the decrease in population density, the telomere lengths also decrease. After their critical senescence point, the telomeres elongate again and give a pattern that is associated with type II survivors.

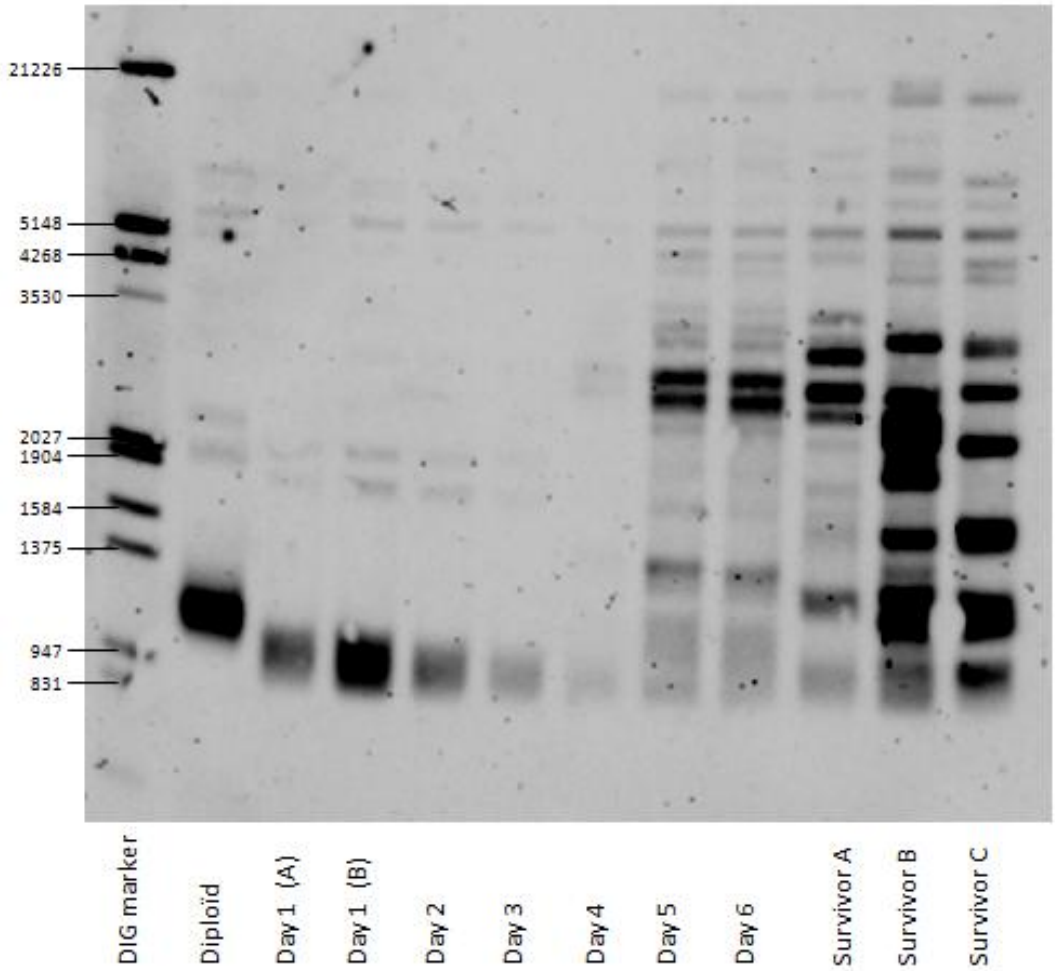


Figure 9 Southern blot with daily sample of the *est2ΔURA3 ARG4 lys2Δ can1-100* senescence assay. Daily collected samples are digested with the digestion enzyme XhoI, run on an agarose gel, transferred to a blotting membrane and hybridized with a digoxigenin labeled probe. Due to problems during the collections of samples at day 1, this day has samples from two different cultures.

Chromatin remodeling genes strains

The second project is based on the role of chromatin remodeling genes during senescence and survivors formation. Six chromatin remodeler genes have been selected and their role during senescence and survivor formation have been characterized using the liquid senescence assay method.

Strain formation

To explore the effect of the deletion of the selected chromatin remodeling genes (*XXX*) in telomerase negative yeast, the genes and telomerase are deleted in the strains. Telomerase is inactivated by replacement of the EST2 gene by an URA3 cassette and the chromatin remodeling genes are deleted by replacement with a KanMX cassette. We dissected the *EST2/est2ΔURA3 XXX/xxxΔKanMX* diploid strains and selected for the haploid *est2ΔURA3 xxxΔKanMX* strains. From the 6 mutants, 4 strains segregated in the expected ratios, table 6. However, 2 strains did not segregate as expected: *EST2/est2ΔURA3 SWI3/swi3ΔKanMX* and *EST2/est2ΔURA3 SNF5/snf5ΔKanMX*, table 7. Also, we did not observe haploid spores containing the *swi3* or the *snf5* deletion. These observations led to the idea that the deletion of snf5 and swi3 might be lethal. Which would explain our observations shown in table 7.

* *XXX* refers to all of the six chromatin remodeling genes. *XXX*: *ASF1*, *SWI3, SNF5*, *SET2*, *SIR2* and *IES4*. *xxxΔKanMX*: *asf1ΔKanMX*, *swi3ΔKanMX*, *snf5ΔKanMX*, *set2ΔKanMX*, *sir2ΔKanMX* and *ies4ΔKanMX*.

Liquid senescence assay

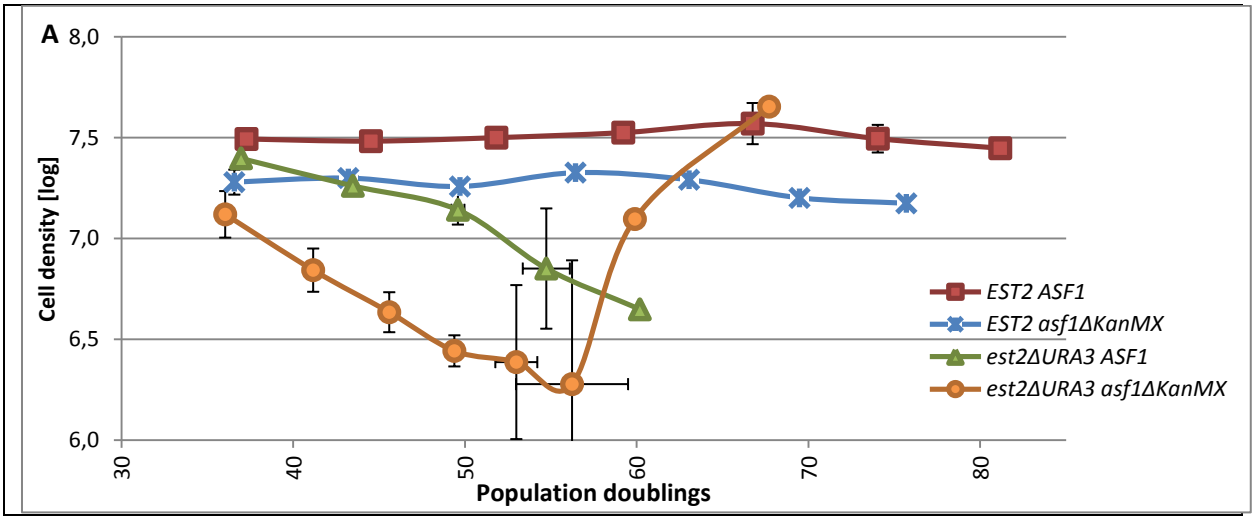
All four acquired haploid strains from a diploid strain are followed during their senescence and recovery phase in liquid growth media. Every genotype is follow twice with spores from different tetrads. As expected, wild-type and single *asf1* deleted strains do not senescence and reach every day the same population doublings, figure 10A and supplementary figure 1A. However, the number of daily population doublings of this latter one is slightly smaller than of a wild-type population (*EST2 ASF1*: 7.51±0.050 PD/24h; *EST2 asf1ΔKanMX*: 7.26±0.049 PD/24h). In the telomerase negative strains the population density of *est2ΔURA3 ASF1* decreases with $9.6 \cdot 10^5$ cells/mL per population doubling and the population doubles 7.2 times every 24 hours. For *est2ΔURA3 asf1ΔKanMX* this is a decrease of $5.2 \cdot 10^5$ cells/mL per population doubling and 6.8 population doublings every 24 hours. Hereafter, the double deletion population restores itself to wild-type density at a rate that is higher than normal telomerase negative populations (an increase of $3.8 \cdot 10^6$ cells/mL per population doubling (*est2Δ asf1Δ*) instead of $1{,}8 \cdot 10^6$ cells/mL per population doubling (*est2Δ ASF1*)). Due to the inability to collect the data points of the *est2ΔURA3 ASF1* strain until the end of the assay, these population restoration results are based upon the average of all telomerase negative strains. These strains give, because of the same genotype, similar data.

Table 6. Distribution of genotypes in dissected tetrads; *ASF1/asf1ΔKanMX*, *EST2/est2ΔURA3 SET2/set2ΔKanMX*, *EST2/est2ΔURA3 SIR2/sir2ΔKanMX* and *EST2/est2ΔURA3 IES4/ies4ΔKanMX*. All four possible genotypes, the number of times they appeared and the amount of isolates with this genotype that are expected to be found are listed. Dissected tetrads, respectively: 10, 15, 12, 9. *est2ΔURA3* is selected on SD-ura, *geneΔKanMX* is selected on YPD+G418.

	ASF1		***SET2***		***SIR2***		***IES4***	
Genotype	**n**	**Expected n**	**n**	**Expected n**	**n**	**Expected n**	**n**	**Expected n**
EST2 GENE	7	10	16	15	13	12	7	9
EST2 geneΔkanMX	13	10	14	15	11	12	11	9
est2ΔURA3 GENE	13	10	14	15	11	12	11	9
est2ΔURA3 geneΔKanMX	7	10	16	15	13	12	7	9
Total	40	40	60	60	48	48	36	36

Table 7. Distribution of genotypes in dissected tetrads; *EST2/est2ΔURA3 SWI3/swi3ΔKanMX* and *EST2/est2ΔURA3 SNF5/snf5ΔKanMX*. All four possible genotypes, the amount of not grown isolates and the number of times they appeared are listed. Together with the amount of isolates with this genotype that are expected to be found and the alternative expected amount isolates. The alternative expected is based upon the idea that haploid strains containing a swi3ΔKanMX or a snf5ΔKanMX are not viable. Dissected tetrads, respectively: 19 and 23. *est2ΔURA3* is selected on SD-ura, *geneΔKanMX* is selected on YPD+G418.

	SWI3			***SNF5***		
Genotype	**n**	**Expected n**	**Alternative expected n**	**n**	**Expected n**	**Alternative expected n**
EST2 GENE	19	19	19	14	20	20
EST2 geneΔkanMX	0	19	0	0	20	0
est2ΔURA3 GENE	19	19	19	26	20	20
est2ΔURA3 geneΔKanMX	0	19	0	1	20	0
Not grown	38	0	38	39	0	40
Total	76	76	76	80	80	80



Also, wild-type and *EST2 set2ΔKanMX* strains double themselves every day to a similar population density as expected, figure 10B and supplementary figure 1B. The population density of *est2ΔURA3 set2ΔKanMX* decreases less than a normal telomerase negative population, at critical senescence point the population density after 24 hours is still $1.4 \cdot 10^7$ cells/mL in contrast to the average $5.5 \cdot 10^6$ cells/mL in a normal telomerase negative strain. Also, the survivor rate formation is lower than that of a normal telomerase negative population and the population density is already restored after four days, which in most *est2Δ* populations takes six or seven days.

Expected as well is, that *EST2 sir2ΔKanMX* doubles itself every day to a similar population density as a normal wild-type population, figure 10C and supplementary figure 1C. Further, based upon the average of both spores, the rate at which the *est2ΔURA3 sir2ΔKanMX* populations senesce seems to be higher than the *est2ΔURA3 SIR2* populations (a decrease of $1.2 \cdot 10^6$ cells/mL per population doubling instead of $5.0 \cdot 10^5$ cells/mL per population doubling). However, the *est2ΔURA3 sir2ΔKanMX* average is very influenced by the observed values of one of the isolates between day 1 and 2. At this point, the senescence rate is -0.082 cell density [log]/PD while during the rest of the senescing period this rate is -0.039 cell density [log]/PD, which is comparable to the other isolate of this genotype, which has a senescence rate of -0.037 cell density [log]/PD over the whole senescing period. Also, *est2ΔURA3 SIR2* have this similar senescence rate during this period: -0.032 and -0.038 cell density [log]/PD. Furthermore, the rate at which the population density is restored is similar for both telomerase negative strains (an increase of $1.3 \cdot 10^6$ cells/mL per population doubling (*est2ΔURA3 SIR2*) and $1.4 \cdot 10^6$ cells/mL per population doubling (*est2ΔURA3 sir2ΔKanMX*)).

At last, both the wild-type and the *EST2 ies4ΔKanMX* strain do not senesce and reach every day the same population doublings, just as expected, figure 10D and supplementary figure 1D and in contrast to the telomerase negative strains. Overall the two strains have a similar senescence rate and the *est2ΔURA3 ies4ΔKanMX* strain has a slightly smaller restoring rate (an increase of $2.6 \cdot 10^6$ cells/mL per population doubling (*est2ΔURA3 IES4*) and an increase of $2.0 \cdot 10^6$ cells/mL per population doubling (*est2ΔURA3 ies4ΔKanMX*). However, there is a big variation in senescence curves within the strains. Separately, the *est2ΔURA3 ies4ΔKanMX* strains have a senescence rate of -0.028 and -0.085 cell density [log]/PD, and the *est2ΔURA3 IES4* strains of -0.042 and -0.072 cell density [log]/PD (respectively a decrease of $7.7 \cdot 10^5$, $1.5 \cdot 10^6$, $1.3 \cdot 10^6$ and $2.2 \cdot 10^6$ cells/mL per population doubling). The first double mutants strain has its critical senescence points between day 3 and 4, for the other strain this is between day 5 and 6, for both *est2ΔURA3 IES4* this is between day 3 and 4. Also, the rate at which the populations are restored varies within the strains. *est2ΔURA3 ies4ΔKanMX* restores in a rate of 0.111 or 0.140 cell density [log]/PD, and the *est2ΔURA3 IES4* in a rate of 0.066 or 0.127 cell density [log]/PD (respectively an increase of $3.4 \cdot 10^6$, $3.3 \cdot 10^6$, $2.0 \cdot 10^6$ and $4.6 \cdot 10^6$ cells/mL per population doubling).

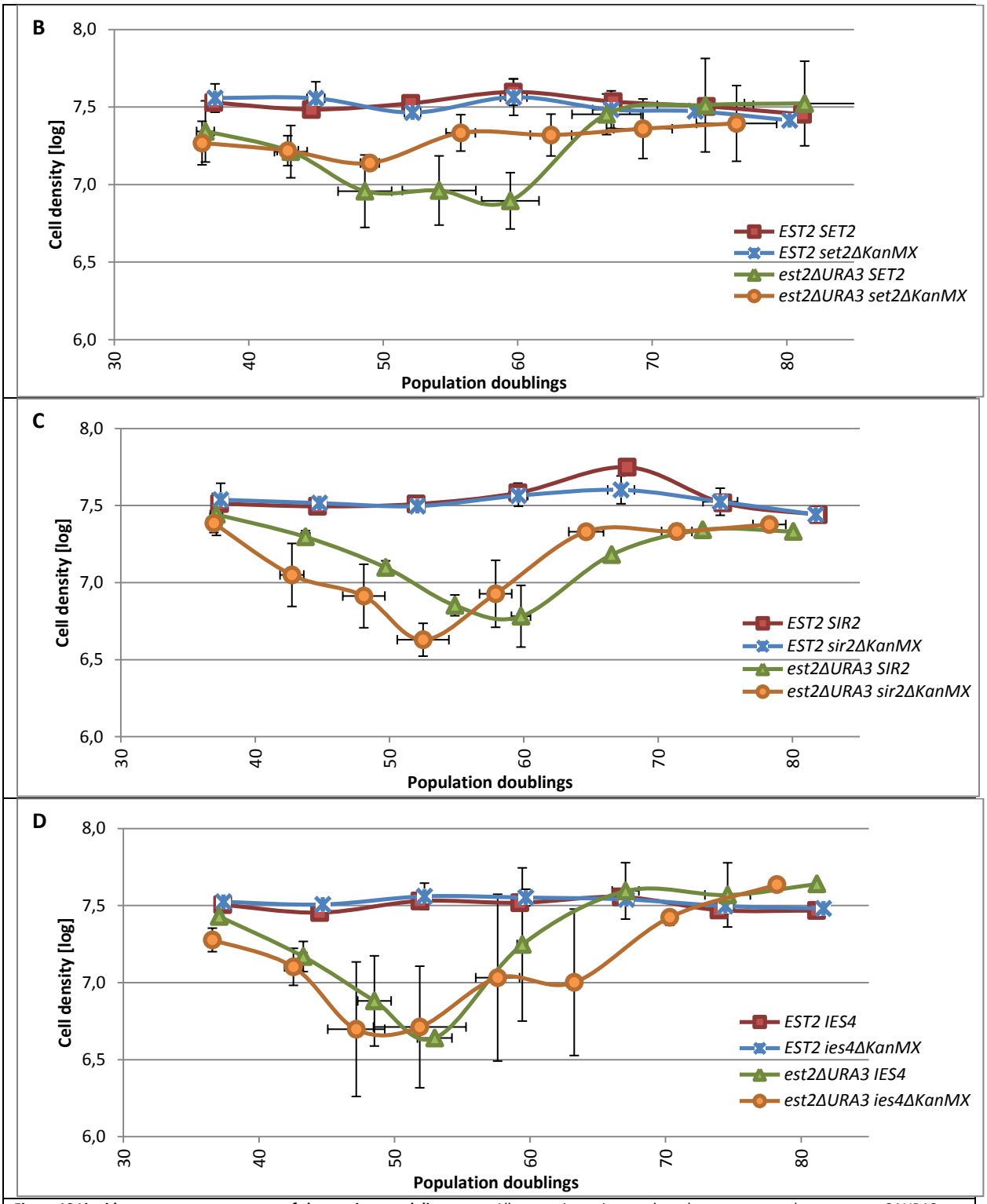


Figure 10 Liquid senescence assay curves of chromatin remodeling genes. All measuring points are based upon two samples, except *est2ΔURA3 ASF1* which has only one measuring point at day 5 and days 6-8 no measuring points and *EST2 asf1ΔKanMX*, *EST2 SET2*, *EST2 set2ΔKanMX*, *EST2 SIR2*, *EST2 sir2ΔKanMX* and *est2ΔURA3 SIR2* which all have only one measuring point at days 6-8. These points were lost due to contamination. **A.** *est2ΔURA3 ASF1*has a senescence rate of -0.033 (decrease of $9.6 \cdot 10^5$ cells/mL per population doubling). *est2ΔURA3 asf1ΔKanMX* has a senescence rate of -0.050 (decrease of $8.0 \cdot 10^5$ cells/mL per population doubling) and a survivor formation rate of 0.224 (increase of $3.8 \cdot 10^6$ cells/mL per population doubling) **B.** *est2ΔURA3 SET2* has a senescence rate of -0.032 (decrease of $1.1 \cdot 10^6$ cells/mL per population doubling) and a survivor formation rate of 0.078 (increase of $2.9 \cdot 10^6$ cells/mL per population doubling). *est2ΔURA3 set2ΔKanMX* has a senescence rate of -0.010 (decrease of $4.2 \cdot 10^5$ cells/mL per population doubling) and a survivor formation rate of 0.029 (increase of $1.2 \cdot 10^6$ cells/mL per population doubling). **C.** *est2ΔURA3 SIR2* has a senescence rate of -0.033 (decrease of $1.2 \cdot 10^6$ cells/mL per population doubling) and a survivor formation rate of 0.059 (increase of $1.3 \cdot 10^6$ cells/mL per population doubling). *est2ΔURA3 sir2ΔKanMX* has a senescence rate of -0.046 (decrease of $5.0 \cdot 10^5$ cells/mL per population doubling) and a survivor formation rate of 0.058 (increase of $1.4 \cdot 10^6$ cells/mL per population doubling). **D.** *est2ΔURA3 IES* has a senescence rate of -0.050 (decrease of $1.4 \cdot 10^6$ cells/mL per population doubling) and a survivor formation rate of 0.067 (increase of $2.6 \cdot 10^6$ cells/mL per population doubling). *est2ΔURA3 ies4ΔKanMX* has a senescence rate of -0.053 (decrease of $1.2 \cdot 10^6$ cells/mL per population doubling) and a survivor formation rate of 0.042 (increase of $2.0 \cdot 10^6$ cells/mL per population doubling).

Senescence assay on plates

To investigate the role of the selected chromatin remodeler genes on senescence rate and type I survivor formation, we preformed senescence assays on plates. Type I survivors appear more frequent than type II survivors, therefore performing the assay on plates gives an advantage to select for type I survivors and to investigate the effect of the genes on type I survivor formation.

All the spores of the chromatin remodeling strains chosen for the liquid senescence assay, are also grown on YPD plates, re-streaked to a new plate every 48 hours and scored based on the amount of colonies and colony growth in this time period, figure 11. This is to follow their decrease in cell density and their recovery back to original density. Almost all telomerase positive strains (*EST2 GENE* and *EST2 geneΔKanMX*) remain growing normally as expected and do not have a score lower than a four on any of the days, table 8 and supplementary figure 2A-D. An exception is *EST2 asf1ΔKanMX*, which mainly scored three or four. The telomerase negative senesce and recover as expected. These strains initially grow similar to telomerase positive strains, but after every re-streak their ability to grow and divide reduces, leading to less and smaller colonies. However, after the fourth streak they start to grow again and every streak the colonies start to look more like telomerase positive colonies.

At the first streak, *est2ΔURA3 asf1ΔKanMX* colonies already appear to look more senesced than normal telomerase negative strains. Hereafter, their ability to divide and grow reduces dramatically to a point where the cells seemed to have died. However, the cells start growing and dividing again, and restore to a growth rate and colony size similar to their initial appearance. Though, the cell growth was not restored to normal wild-type growth (score 5).

Initially the *est2ΔURA3 set2ΔKanMX* strains are less capable of growing up till maximal telomerase positive growth (score 5). Their growth at the first time point is scored as 3-4 and at the second streak it is dropped to 3. In contrast to normal telomerase negative strains, their growth does not reduce further, their colony growth and division rate stays at a rate that is scored as 3. However, around the fifth re-streaking time point, the spores slightly increase their growth and division rate, therefore scored slightly higher during the last time points.

The *est2ΔURA3 sir2ΔKanMX* and *est2ΔURA3 ies4ΔKanMX* strains reduce in growth and recover growth in a similar fashion as a telomerase negative strain.

Conclusion

No conclusions can be made for the influence of the deletions on survivor type formation. This is because the data needed for this conclusion should be provided by southern blot analysis and due to lack in time, these experiments have not been done. However, we can conclude on their influence on senescence rate and survivor formation rate.

As expected, the wild-type and telomerase positive single deletion strains do not senesce and at every time point they grow to a density which is similar as wild-type yeast. However, the deletion of *asf1* does not induce senescence but it seems to negatively influence the rate at which cells divide and grow. Therefore, leading to a similar but smaller density at every time point.

Table 8 Scoring of senescence on plates in chromatin remodeling strains. All strains are followed during their senescence and survivor formation on YPD plates. Streaks are scored with numbers between 0 and 5: 0 dead, 1 severe senescence, 5 normal (wildtype) growth. Shown scorings are based on eight samples for *EST2*, 8 samples for *est2ΔURA3* and each 2 samples for *EST2 asf1ΔKanMX*, *est2ΔURA3 asf1ΔKanMX*, *est2ΔURA3 set2ΔKanMX*, *est2ΔURA3 sir2ΔKanMX* and *est2ΔURA3 ies4ΔKanMX*.

	Time point						
Genotype	**1**	**2**	**3**	**4**	**5**	**6**	**7**
EST2	4.9	5.0	4.9	4.8	5.0	4.3	4.9
EST2 asf1ΔKanMX	4.0	4.5	3.0	4.0	4.0	3.0	3.0
est2ΔURA3	4.0	2.8	1.0	1.9	2.9	3.1	3.9
est2ΔURA3 asf1ΔKanMX	3.0	3.0	1.0	0.0	1.0	2.0	2.5
est2ΔURA3 set2ΔKanMX	3.5	3.0	3.0	3.0	3.5	3.5	4.0
est2ΔURA3 sir2ΔKanMX	4.0	2.0	1.5	1.5	3.0	3.0	3.0
est2ΔURA3 ies4ΔKanMX	4.0	2.5	1.5	2.0	3.5	3.5	4.5

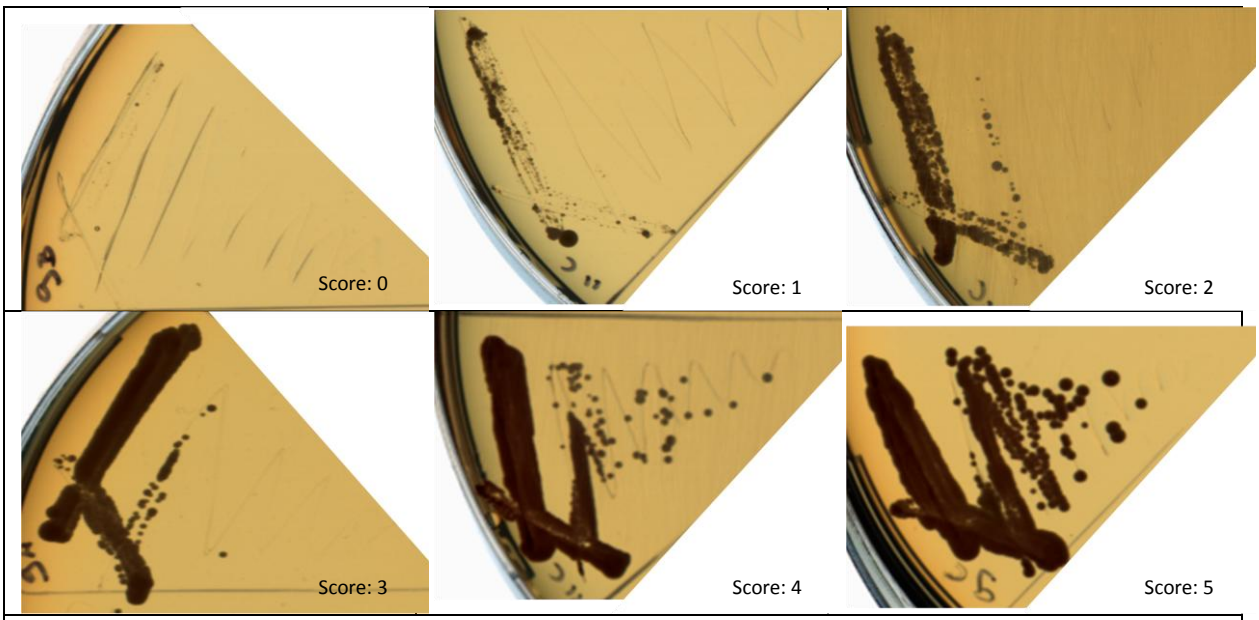


Figure 11 Scoring of senescence assay on plates. Examples of colony growth and their scoring.

Also the telomerase negative single deletion strains behave as expected. Initially they are able to grow at a similar rate as wild-type cells. However, after the first time point they start to senesce until they reach a critical senescence point around day 4 (liquid) and streak 3 (plates). Hereafter, their ability to grow and divide increases again to a point where they behave as wild-type yeast.

The double deleted strains *sir2* and ies4, have a similar senescence curve. Even though the liquid senescence assay data gives mixed signals. The senescence assay on plates shows that these two strains senesce in a similar manner as single deleted telomerase negative strains. Therefore, these deletions do not seem to influence the senescence rate and survivor formation rate in *Saccharomyces cerevisiae*.

In contrast, the double deleted strains *asf1* and *set2* do not behave as telomerase negative strains. The telomerase negative *asf1* strain immediately grows and divides less than telomerase negative strains. Further, also the senescence rate is higher, the critical senescence point is at a lower density and the survivor formation rate is higher than telomerase negative yeast. The telomerase negative *set2* strain also reaches a lower density at the first time point. However, the strain does not seem to start senescing, during the whole assay the strain reaches a similar density at every time point. This suggests that both deletions are involved in the senescence of budding yeast.

Discussion

Our results show that the *arg4Δ* deletion, *lys2Δ* deletion, and restoration of the *CAN1* gene do not alter the senescence and survivor formation of telomerase negative yeast. However, it seems to affect the mating and sporulation capabilities of EST2*/EST2 arg4Δ/arg4Δ lys2Δ/lys2Δ CAN1/CAN1* and *EST2/est2Δ arg4Δ/arg4Δ lys2Δ/lys2Δ CAN1/CAN1* diploids. Initially, it was thought that the *arg4* deletion, in combination with the *lys2* deletion, influences the capability to mate and sporulate in W303 strains. Though this only occurs in this strain, while in other *Saccharomyces cerevisiae* strains these problems are not detected, e.g. in the strains BF264-15D, BY4742, BY4743 and YMJ38[40-44]. However, Chen et al. were able to produce an *arg4ΔKAN lys2ΔNAT CAN1* strain from a W303-1A background without mentioning mating and sporulation efficiency problems[45]. This suggests that this group did not obtain their haploid strain from an *arg4Δ/arg4Δ lys2Δ/lys2Δ CAN1/CAN1* diploid and therefore did not encounter this complication. It might also be that the double *arg4* deletion in combination with *TRP1*, *ADE2*, *SML1* or *RAD53* (instead of *trp1-1*, *ade2-1*, *sml1::HIS3* or point mutations in *RAD53*), and possibly also involving the *lys2* deletion, alter the mating and sporulation efficiency of W303[45]. However, this latter option seems unlikely as wild-type genes normally do not dramatically inhibit essential mechanisms like reproduction.

Further, the deletions and restoration we made also do not seem to alter senescence and survivor formation, regardless of the fact that the tested strains do not show similar senescence assay curves. However, the strains do show similar changes in morphology. The differences in the curves are likely caused by the use of spores that are not clonal. Because of this, the length of the shortest telomere and the chromosomal location of this shortest telomere at the beginning of the assays can differ between the spores. Further, differences can occur due to telomerase and which telomeres this enzyme has elongated when the spore was still part of the diploid strain. These differences in the spores can influence the senescence rate, the reduction in population density and the day of critical senescence, therefore leading to slightly different curves. However, if one clonal spore was followed multiple times, the initial telomere lengths are more or less identical and similar curves are expected to appear.

In the end, although we were not able to perform SILAC, we were able to form the strain that can be used in combination with heavy-labeled arginine and lysine to quantify the proteome at different time points during the senescence assay. The assays also indicate at which days the wanted time points occur: pre-senescence cells at the first collection day, cells with critically short telomeres at the day the curve is at the lowest point (to prevent too many death cells in the sample it is collected the day before this measured critical point), and survivor cells at the day the cell density does not increase further and reaches wild-type values. With this we hope to see differences in protein abundance between the different stages. However, it is possible that there are no (noticable) abundance changes, because the changes are too small to observe or because ALT is initiated by another process, for example by changes in localization of one or multiple proteins.

For the chromatin remodeler project, we were able to analyze the effect of the absence of four chromatin remodeler genes, *asf1Δ*, *set2Δ*, *sir2Δ* and *ies4Δ*, on senescence rate. We were unable to analyze *swi3Δ* and *snf5Δ*, as we were not able to generate haploid strains with these deletions. However, our inability to generate these strains is consistent with previous observation made by other researchers. These researchers have mentioned for both mutants that diploids with one of these mutations fail to sporulate efficiently[46-48]. This enlightens why we were unable to acquire these strains, and it matches with the findings that the proteins of the SWI/SNF complex are involved in the transcriptional regulation of many genes in baker's yeast[27, 29, 49]. Therefore, it is likely that the inactivation of subunits of the SWI/SNF complex affects processes like mating and sporulation.

Of the four examined genes, the *sir2* and *ies4* mutants did not differ from strains that are only telomerase negative, even though otherwise is expected. This is because *SIR2* is involved in inter alia the regulation of recombination[32, 33]. Hence, it is hypothesized that the deletion of this gene, and therefore the absence of its encoded protein, could alter a recombination-mediated mechanism like ALT. Thus, a strain containing *sir2Δ* could lead to less recombination, less elongation of the shortest telomeres, and as a result, to less or even no formation of survivors. On the other hand, the deletion might positively affect recombination, elongating telomeres when they are not yet critically short or elongate more telomeres in more yeast cells and because of that cause an earlier and/or faster recovery of the population. Further, the sir2 protein also facilitates transcriptional silencing at telomeres[32, 33]. Hence, the absence of this protein might increase transcription of genes located at or near the telomeres and influence the activation of genes involved in the initiation of ALT and survivor formation.

Less is known about the ies4 protein, but the protein is suggested to be involved in the DNA damage signaling pathway. This pathway is initiated when telomeres become critically short and are recognized as double strands breaks. The pathway responds by inhibiting the cell cycle and initiating senescence or apoptosis[35, 36, 50]. This implies that the absence of this protein could affect this pathway and therefore might influence senescence.

Based upon these ideas, it was expected that the deletion of *sir2* and *ies4* would change the senescence and/or survivor formation of telomerase negative yeast. However, no effects on the senescence rate, reduction in population density, time point of critical senescence or survivor formation rate, have been seen. This suggests that these two genes do not influence the senescence of telomerase negative *Saccharomyces cerevisiae*. However, even though it is not seen in the liquid senescence assay and the senescence assay on plates, the genes might still be involved in the survivor formation, for example by changing the occurrence of a survivor type.

In contrast, the deletion of the two other chromatin remodeler genes, *asf1* and *set2* in telomerase null strain, do cause differences in the senescence rate compared to the single telomerase null strains. The *asf1* deletion negatively affects the growth rate of both telomerase positive and negative strains and leads to an increased senescence rate in the latter strain. The effect of the *asf1* deletion on the growth of a population is also shown by other researchers and they found that this deletion also reduces the life span of single yeast cells by 65%[51-53]. Because of this, it seems more likely that the increased senescence rate that is seen in the double mutant is caused by an additional decrease in cell growth, next to the decrease caused by the absence of telomerase, than that *ASF1* actually influences senescence. Further, *ASF1* derepresses genes that initiate the DNA damage response[52, 54].

Hence, due to the deletion of this gene, the DNA damage response genes might continue to be repressed when telomeres become critically short. These cells might not immediately go into cell cycle arrest and accumulate DNA damage before they eventually undergo apoptosis, senescence or become cancerous. Consequently, the population density decreases more than in single deleted telomerase negative strains, which is fitting with our findings. At last, the survivor formation rate increases in the double mutants twice as much as in single mutants. Thus, the *asf1Δ* telomerase negative survivors grow and divide twice as fast as normal telomerase negative strains. This suggests that the *asf1* deletion, even though initially inhibiting cell growth, stimulates the growth of survivors, and that *ASF1* reduces this elevated survivor growth.

In the end, we were able to complete the first phases of the methods to answer our questions. With this we could optimized the protocols and we obtained the first data. However, the study has to be finished to see if the proteome changes between pre-senescence, senescence and survivor cells and if the chromatin remodeler genes are (also) involved in survivor formation. With these results, we might come one step closer to understanding cancer formation. However, more research has to be done to unravel the whole molecular mechanism behind the onset of cancer cells that appear due to ALT. More knowledge might lead to a better screening, a treatment and/or cure for patients with cancer that restores its telomeres via ALT.

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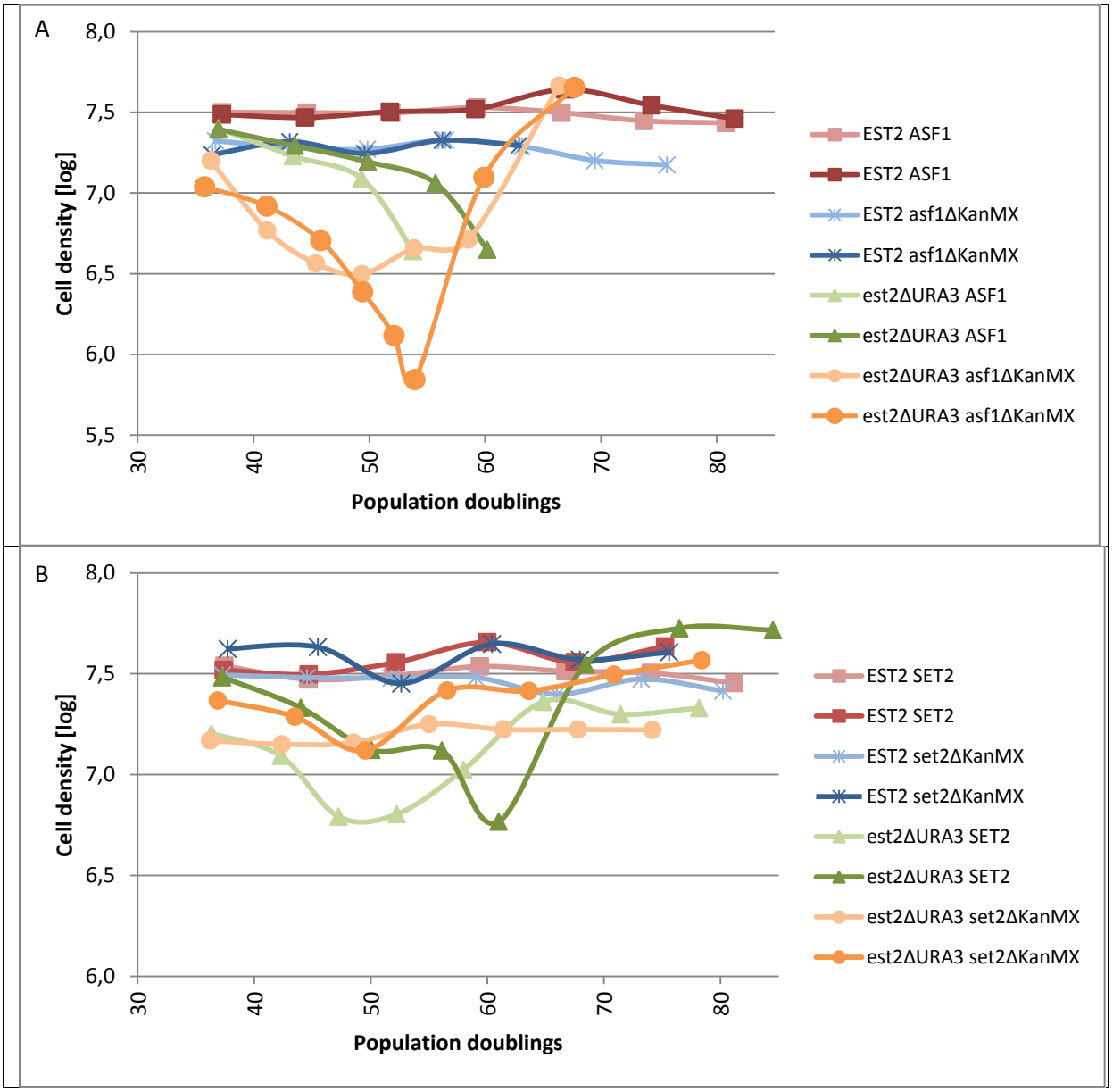
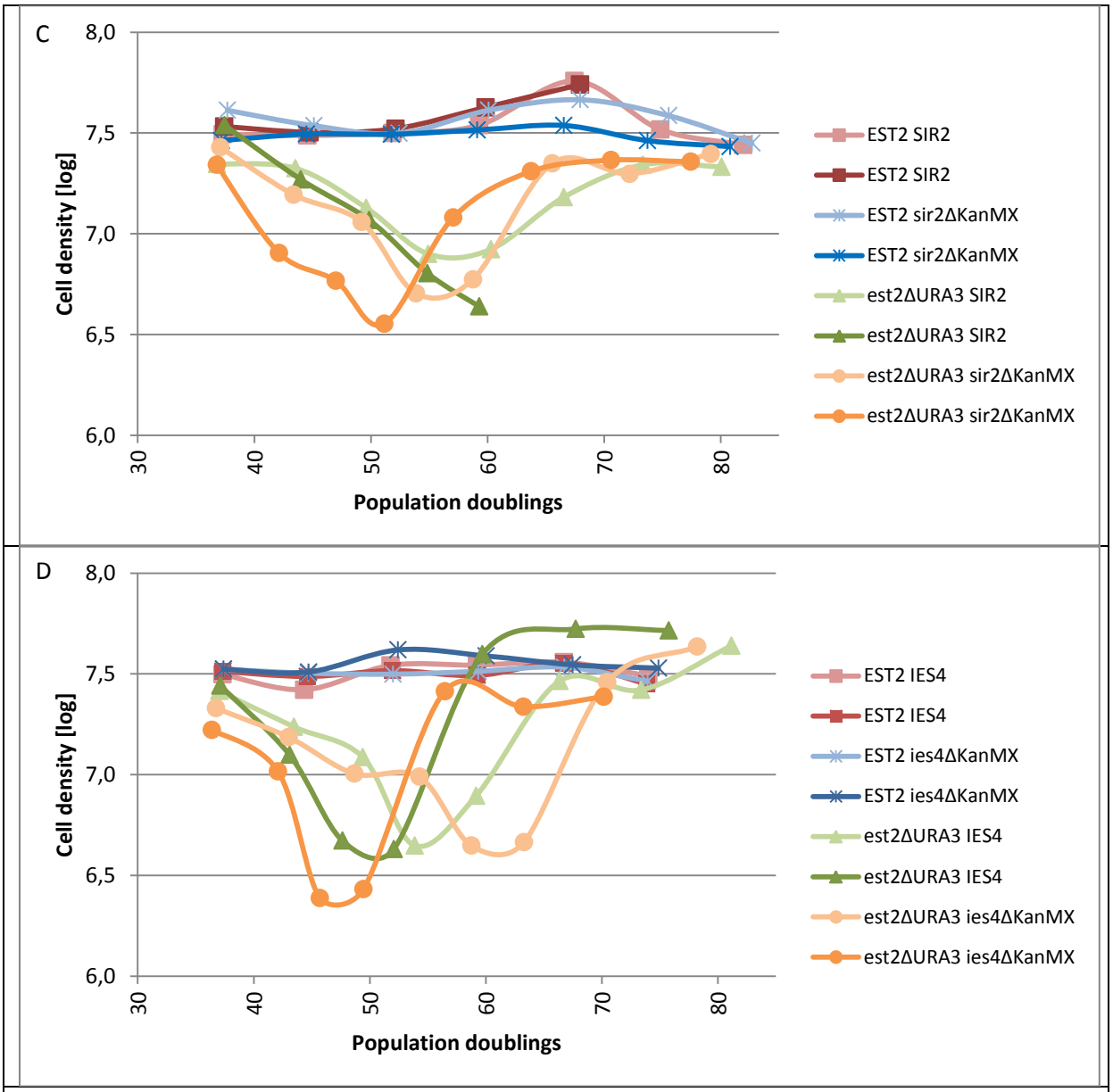
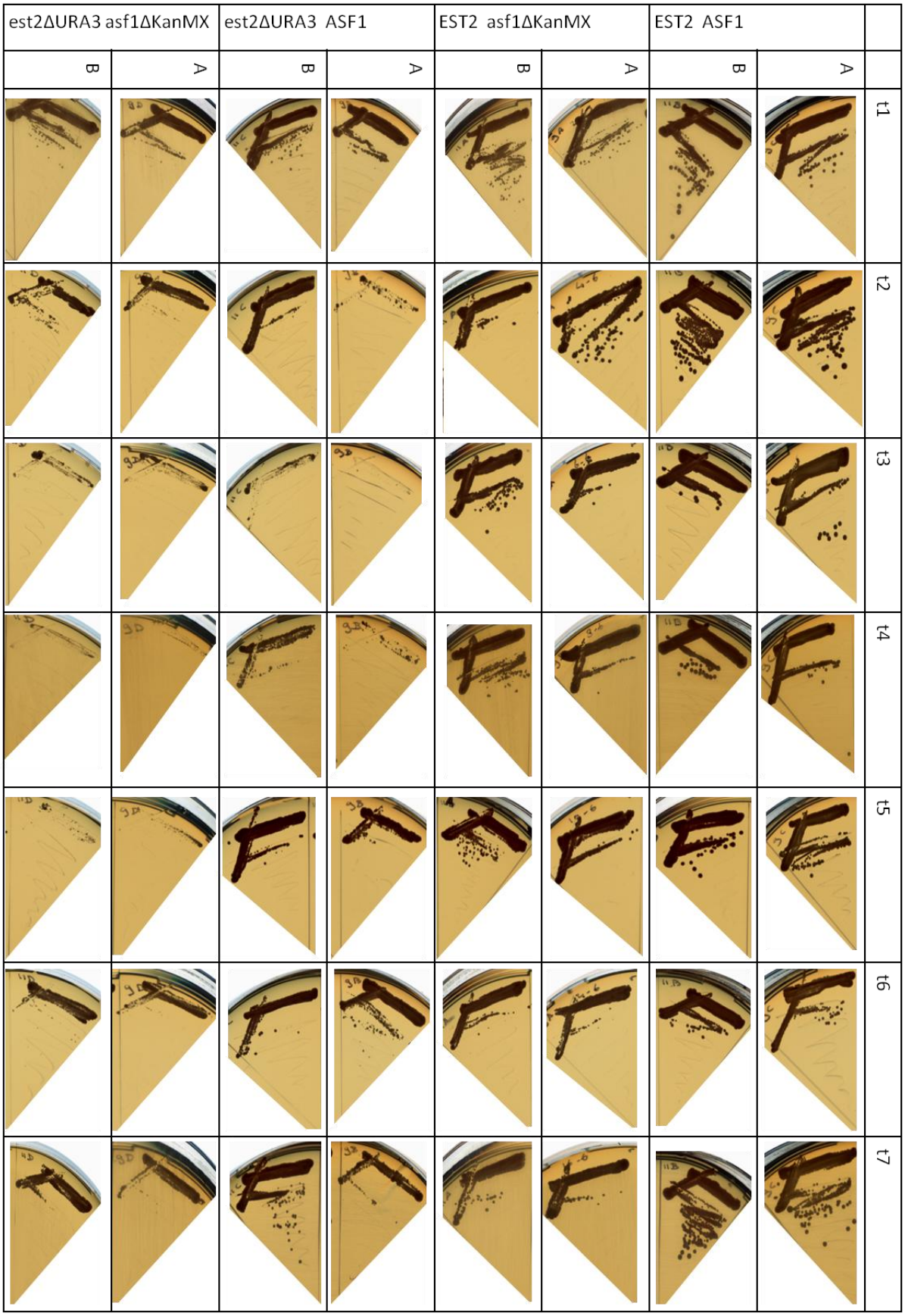
Supplementary





Supplementary figure 1 Liquid senescence assay curves of chromatin remodeling genes. All used tetrads contained isolates with four different genotypes. All these genotypes are diluted to $2 \cdot 10^5$ cells/mL in 5 mL YPD and cultured for 24 hours at 30 °C. Thereafter, the cell density is measured and samples are collected. Two dissected tetrads are followed for every strains; **A.** *EST2/est2ΔURA3 ASF1/asf1ΔKanMX*, **B.** *EST2/est2ΔURA3 SET2/set2ΔKanMX*, **C.** EST2*/est2ΔURA3 SIR2/sir2ΔKanMX* and **D.** *EST2/est2ΔURA3 IES4/ies4ΔKanMX*.

A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est2ΔURA3 asf1ΔKanMX
A
B
A
B
A
B
A
B
t1
t2
t3
t4
t5
t6
t7

B

EST2 SET2
EST2 set2ΔKanMX
est2ΔURA3 SET2
est2ΔURA3 set2ΔKanMX
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B
A
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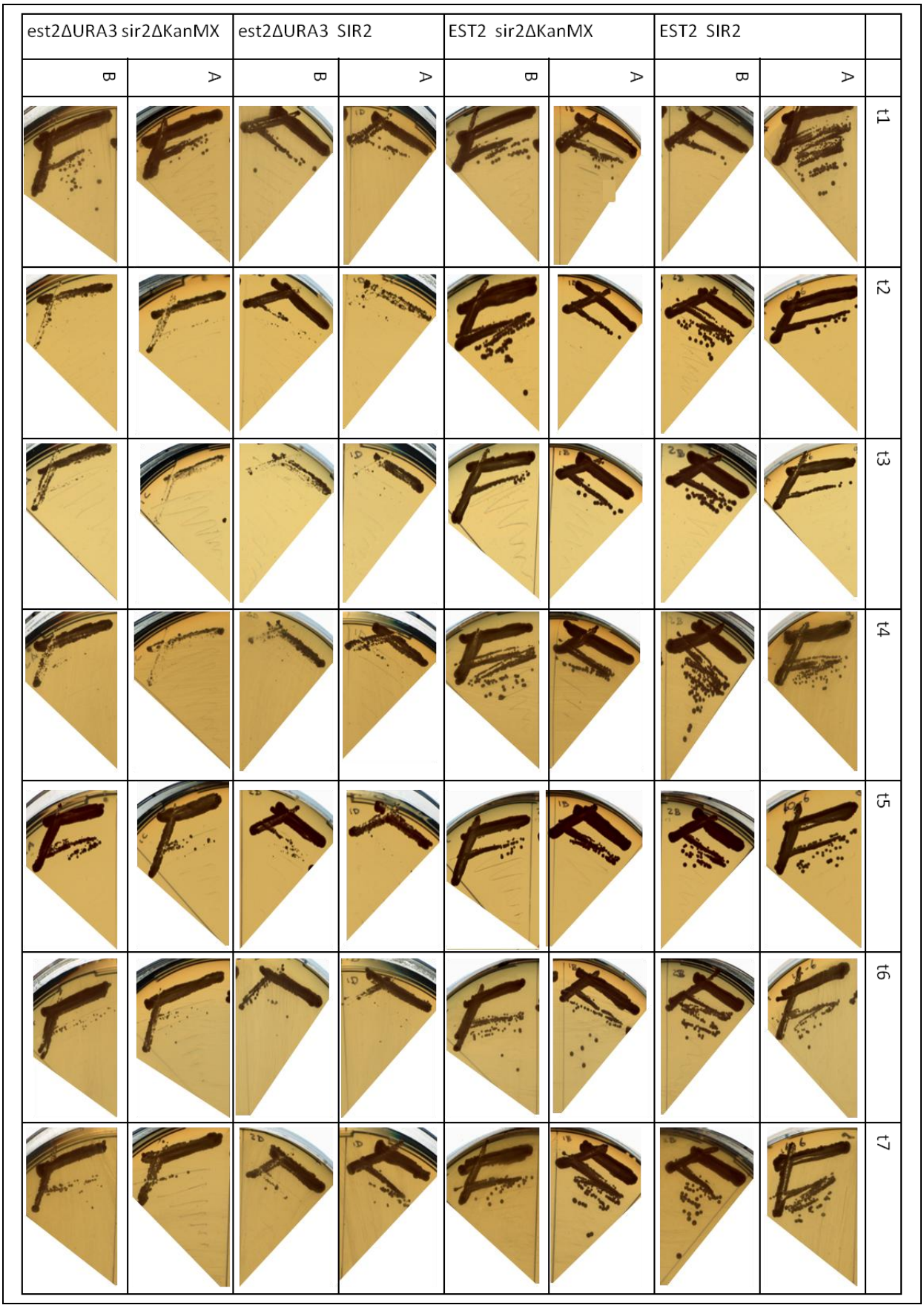
C


est2ΔURA3 sir2ΔKanMX
est2ΔURA3 SIR2
EST2 sir2ΔKanMX
EST2 SIR2
B
A
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A
t1
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t3
t4
t5
t6
t7

D

Supplementary figure 2 Senescence assay of chromatin remodeling genes on plates. All used tetrads contained isolates with four different genotypes. All these genotypes are streaked for single colonies on a YPD plate and grown for 48 hours at 30 °C. Thereafter, similar amounts of cells are picked, re-streaked on a fresh YPD plate, grown and repeated until the isolate is back to initial growth. Two dissected tetrads are followed for every strains; **A.** *EST2/est2ΔURA3 ASF1/asf1ΔKanMX*, **B.** *EST2/est2ΔURA3 SET2/set2ΔKanMX*, **C.** EST2*/est2ΔURA3 SIR2/sir2ΔKanMX* and **D.** *EST2/est2ΔURA3 IES4/ies4ΔKanMX*.