Povezanost signalnog puta NMD s alternativnim produljenjem telomera i preživljenjem stanica Saccharomyces cerevisiae

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Investigating the link between nonsense-mediated mRNA decay pathway and survivor formation in *Saccharomyces cerevisiae*

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Povezanost signalnog puta NMD s alternativnim produljenjem telomera i preživljenjem stanica *Saccharomyces cerevisiae*

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Degradacija molekula mRNA s besmislenim mutacijama (NMD) kontrolni je mehanizam koji selektivno razgrađuje mRNA s preuranjenim završnim kodonom (PTC). Istraživanja pokazuju da NMD regulira i koncentraciju mnogih mRNA koje ne sadrže PTC te da je time uključen u kontrolu određenih staničnih procesa, konkretno nastanak preživjelih stanica tipa II. Ovo istraživanje ispituje ovisnost nastanka takvih stanica o ekspresiji gena *UPF1*, *UPF2* i *UPF3*, koji čine NMD u *S. cerevisiae* te gena *EBS1*, koji literatura povezuje s NMD. Također se istražuje direktna veza između NMD i nastanka preživjelih stanica tipa II, a kao vjerojatni kandidati su odabrani proteini Stn1 i Rad51. Rezultati pokazuju kako nastanak preživjelih stanica tipa II nije onemogućen pojačanom ekspresijom gena *STN1*. Potrebno je ipak ispitati može li pojačana ekspresija gena *STN1* djelomično ometati nastanak takvih stanica. U mutanata s pojačanom ekspresijom gena *RAD51* nastanak preživjelih stanica spomenutog tipa nije primijećen. Ulogu tog gena kao veze između NMD i formiranja preživjelih stanica tipa II treba, međutim, dodatno ispitati jer je na rezultate mogao utjecati način provođenja eksperimenta. Opažanja opisana u radu podržavaju hipotezu da je NMD uključen u regulaciju nastanka preživjelih stanica tipa II, ali potrebna su dalja istraživanja kako bi se preciznije odredila priroda te veze.

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Ključne riječi: Put degradacije molekula mRNA s besmislenim mutacijama (NMD), preživjele stanice tipa II, gen *UPF1*, gen *UPF2*, gen *UPF3*, gen *STN1*, gen *RAD51*

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Graduation Thesis

Investigating the link between nonsense-mediated mRNA decay pathway and survivor formation in *Saccharomyces cerevisiae*

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Nonsense-mediated mRNA decay (NMD) is a eukaryotic mechanism of RNA surveillance that selectively degrades transcripts containing premature termination codons (PTC). Previous studies suggest that this pathway also regulates the abundance of many cellular mRNAs without a PTC. It is therefore very likely that it surveils certain cellular processes, among them regulation of type II survivor formation. This study tests whether the generation of type II survivors depends on the three genes composing NMD in *S. cerevisiae* – *UPF1*, *UPF2* and *UPF3*. Dependency of this process on *EBS1* is also examined because this gene is supposed to be connected to NMD. Furthermore, the exact connection between NMD and type II survivor formation is studied and two proteins, namely Stn1 and Rad51, are chosen as likely candidates for this link. In the case of *STN1*, results suggest that type II survivor generation is not blocked by overexpression of this gene. Whether it is partially impaired remains to be checked. Type II survivors have not been detected in mutants with *RAD51* overexpression but the results may have been influenced by the way in which the assay was carried out. The observations made in this study enable a general conclusion that NMD is most likely involved in the regulation of type II survivors, while the exact link remains to be discovered.

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Key words: Nonsense-mediated mRNA decay (NMD), type II survivors, *UPF1*, *UPF2*, *UPF3*, *STN1*, *RAD51*

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Abbreviations

NMD	Nonsense-mediated mRNA decay
PTC	Premature termination codon
DSE	Downstream sequence elements
UTR	Untranslated regions
PABP	Poly(A) binding protein
bp	Base pairs
ALT	Alternative Lengthening of Telomeres
ChiP	Chromatin immunoprecipitation
YPD	Yeast extract-peptone-dextrose
YPD+Cu	Yeast extract-peptone-dextrose with added $CuSO_4$
YPGal	Yeast extract-peptone-galactose
gDNA	Genomic DNA
GALpr	Galactose promotor
CUpr	Copper promotor
T_a	Annealing temperature
RT	Room temperature
DO	Dropouts

Contents

1. Introduction

In this thesis I explore the connection between nonsense-mediated mRNA decay and type II survivor formation in the budding yeast *Saccharomyces cerevisiae*. The project described here was performed at the European Research Institute for the Biology of Ageing in Groningen in 2016, in the framework of Dr. Michael Chang's research focusing on telomeres and genome integrity. Relevant information about the key elements in my study has been found in published literature.

1.1. Nonsense-mediated mRNA decay

Cells have evolved several mRNA surveillance mechanisms that are used for the degradation of messenger RNA (mRNA) molecules with mutations. Consequently, the production of proteins with changed structure is prohibited so that no aberrant proteins can confer their toxic effect upon the cell. These mechanisms operate both in the nucleus and in the cytoplasm (Isken et al., 2007). One of the best studied surveillance mechanisms is the nonsense-mediated mRNA decay (NMD) pathway that selectively targets mRNA molecules bearing a premature termination codon (PTC). PTCs, also called premature stop codons, are caused by nonsense mutations which can arise already at the DNA level or as a consequence of mistakes made during transcription or RNA processing. Action of this pathway is confined to the cytoplasm and is closely linked to translation termination (Hug et al., 2016). According to Brogna and Wen (2009), NMD was discovered after it was observed that cells often contain a low amount of mRNAs which are transcribed from the alleles that carry nonsense mutations. It was shown that NMD is evolutionary conserved in eukaryotes, with more NMD factors required for functional response in more complex organisms. Surprisingly, the NMD pathway is also involved in the regulation of gene expression by monitoring the levels of mRNAs that do not contain PTCs. Such important and complex regulation would suggest the need for the presence of a buffering mechanism that would control the magnitude of the NMD response in accordance to specific genetic and/or environmental stimuli (Hug et al., 2016). Although many genes whose expression is controlled in such a way have been discovered, there is little overlap between the sets of such genes in different organisms and no correlation with any specific function (Brogna and Wen., 2009). While mRNAs are directly targeted and degraded by this pathway, expression of some genes is downregulated indirectly. Most likely, some of the target mRNA carry the information necessary

for the production of transcription factors or chromatin remodelers. In their absence, it is logical to assume that expression of their target genes would be affected (Guan et al., 2006).

Another proposed function of the NMD pathway is in alternative splicing, where it could eliminate splice variants that contain PTCs or transcripts that have not been spliced. Nevertheless, studies have not been successful in finding the intimate link between NMD and alternative splicing (McGlincy et al., 2008). This pathway is not solely limited to mRNA, since long non-coding RNAs are also used as substrates in *S. cerevisiae*, *Arabidopsis* and mouse embryonic stem cells (Hurt et al., 2013).

1.2. NMD in *Saccharomyces cerevisiae*

In *S. cerevisiae* three proteins comprise the NMD pathway, namely Upf1 (Nam7), Upf2 (Nmd2) and Upf3. The genes which code for the mentioned proteins are evolutionary conserved and represent a necessity for the operational NMD response (Conti et al., 2005). Upf1 is an ATP-dependent RNA helicase of the SFI superfamily. Together with Upf2 and Upf3 it forms a trimeric complex that binds in the vicinity of a PTC. Eventually, the bound mRNA will be degraded and the emergence of truncated proteins will be prevented. One of the most important requirements for the NMD is to be specific towards PTCs only, while ignoring naturally occurring stop codons. According to Hug et al. (2016), "current models hypothesize that Upf1 is selectively recruited onto or activated on prematurely terminating ribosomes". Aside from their function in unwinding secondary structures, RNA helicases can remain bound to a specific location on the RNA where they serve as "place markers". It is believed that Upf1 clamps an mRNA at a specific position and recruits other proteins that are required for the formation of the degradation machinery (Cordin et al., 2006). In the end, mRNA will be degraded by contributions from both the 5'–3' decapping-dependent exonuclease pathway and the deadenylation dependent 3'–5' exosome pathway. It was shown that Upf1 interacts with the decapping enzyme Dcp2 to promote Xrn1-mediated decay from the 5' end (Muhlrad and Parker, 1997) and with Ski7 to promote exosome-mediated decay from the 3' end (Mitchell and Tollervey, 2003). In their paper, Brogna and Wen (2009) describe two models that depict exactly how mRNAs with PTCs are distinguished. One model focuses on the importance of the downstream sequence elements (DSE) to which NMD

factors bind. Because there are several copies of DSEs across the coding part of an mRNA, the closer the PTC is to the beginning of the gene, the more probable that a DSE will be found downstream of it and that PTC will be recognized. As mentioned in the article, the main problem with this model is that there is no similarity between DSEs found on different mRNAs. The other model is called faux 3'-UTR model and it is applicable to yeast and *Drosophila.* It is based on the observation that mRNAs with prolonged 3' untranslated regions (UTR) are NMD substrates, which emphasizes the importance of distance between PTC and 3' UTR for the recognition of PTC and initiation of NMD response. Distinction between PTC and naturally occurring stop codons is made clear by the interaction between the poly(A) tail and the terminating ribosome (Figure 1). In the case of premature stoppage of the ribosome machinery interaction between the terminating ribosome and poly(A) binding protein (PABP) is prevented and association of NMD factors on the terminating ribosome is enabled. In the other case, translation termination takes place close to the 3' UTR and it is dependent on the interaction between PABP and peptide-release factor eRF3, which is associated with the terminating ribosome. Because PABP interacts with peptide-release factor eRF3, NMD factors cannot associate with the terminating ribosome and such mRNA is not "interpreted" as a substrate for NMD. As an argument for the accuracy of this model, authors explain that if PABP is artificially tethered close to the PTC, such PTC-containing mRNAs will be stabilized.

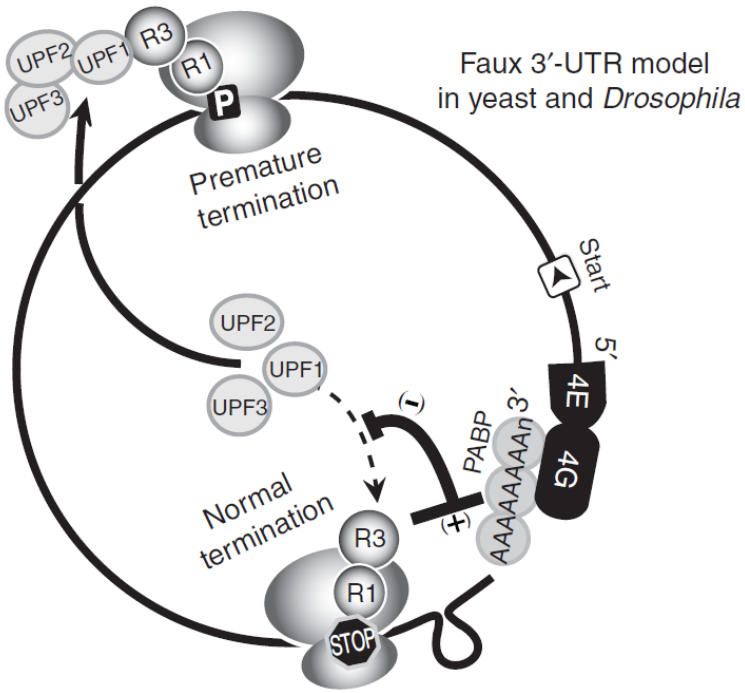

Figure 1. Current NMD model in yeast and *Drosophila*, named faux 3'-UTR model (Brogna and Wen, 2009). Normal termination depends on interaction between PABP, which is located on the poly (A) tail, and peptide-release factor eRF3 associated with the ribosome. In premature termination, interaction between PABP and the terminating ribosome is not possible due to the long distance between the ribosome and the poly(A) tail. Instead, the terminating ribosome interacts with NMD factors.

1.3. Telomeres and survivor formation

Telomeres are specialized nucleoprotein structures that are involved in protecting eukaryotic chromosome ends from fusing together or degradation, hence their importance for genome integrity. In budding yeast telomeres consist of ~350 base pairs (bp) of $TG_{1\text{-}3}/C_{1\text{-}3}A$ repeats followed by a terminal single-stranded (ss) $TG_{1\text{-}3}$ tract called G-overhang (Wellinger et al., 1993). As a consequence of aging, telomeres progressively shorten due to incomplete DNA replication. Progressive shortening of telomeres will in time result in the inability of the cell to divide. Such cells enter a state called replicative senescence, which can be avoided by the action of telomerase or by employment of the machinery required for homologous recombination. Telomerase is a reverse transcriptase that is crucial for the maintenance of telomeric ssDNA. This ribonucleoprotein adds telomeric repeats sequence to the 3' G-overhang by using its intrinsic RNA template (Greider and Blackburn, 1985). Two subunits constitute the core of budding yeast

telomerase – the catalytic subunit Est2 and the RNA template subunit Tlc1. Two additional proteins required for telomerase activity *in vivo* are Est1 and Est3. Est1 interacts with the telomeric ssDNA-binding protein Cdc13 and recruits telomerase to telomere ends. In wild type cells, the telomerase pathway is the more predominant mechanism of telomere elongation as compared to the recombination pathway (Lingner et al., 1997). In telomerase-null cells, however, telomere elongation presumably depends solely on the recombination pathway. This telomerase-independent recombination pathway is termed Alternative Lengthening of Telomeres (ALT). Most often, telomerase deficient yeast strains enter senescence after about 50-100 divisions, when telomeres reach their critical length of approximately 100 bp (Singer et al., 1994). Nevertheless, a few of these senescing cells can bypass the critical point by elongating their telomeres via the Rad52-dependent recombination pathway mentioned above. These cells are referred to as "survivors" and two survivor types, namely type I and type II, can be distinguished by their characteristic telomere patterns. In type I survivors, telomeres are extended by amplification of subtelomeric Y' elements. Along with Rad52, formation of this type of survivors also depends on Rad51, Rad54, Rad55 and Rad57 (McEachern et al., 2006). Type II survivors exhibit sudden elongation of their telomeres by amplification of the $TG_{1–3}$ telomeric repeats, which depends on the Mre11-Rad50-Xrs2 (MRX) complex and Rad59. Type I survivors are generated more frequently in solid-medium-grown cultures than type II: ~90% of newly formed survivors are type I and ~10% are type II. Although type I survivors are formed more often, type II survivors grow at faster rates, eventually exceeding their counterparts in sheer numbers when grown together in liquid culture (Hu et al., 2013).

Recently, other genes involved in survivor formation have been identified. Hu and coworkers (2013) have identified 22 genes that are required for type II survivor generation. Among them there are 4 genes that are involved in NMD, namely *UPF1*, *UPF2*, *UPF3* and *EBS1*. This observation suggests that NMD factors are directly involved in type II survivor formation or maintenance. Although this was reported several years ago, no models have yet been published and the exact molecular mechanism remains to be discovered.

1.4. Involvement of NMD in telomere biology

As mentioned before, the NMD pathway also targets mRNAs which do not contain PTC and it is estimated that levels of around 600 RNAs are regulated in such a way. With regard to the latter, it is plausible to say that the action of the NMD pathway has severe physiological consequences. For example, yeast strains with impaired NMD have alterations in chromosome structure – the number of repeats in telomeres is reduced, the function of kinetochores is altered, gene silencing in silent regions of DNA near telomeres is impaired, etc. (Guan et al., 2006). The fact that yeast cells with impaired NMD show shorter telomere phenotype and faster telomere loss than wild type cells emphasizes the importance of NMD in telomere biology (Holstein et al., 2014). Enomoto and colleagues (2014) report that NMD accelerates the rate of senescence in telomerase deficient yeast strains. Their research has demonstrated that yeast cells with knock-out telomerase and with deletion of either *UPF1* or *UPF2* or *UPF3* show a delayed senescence phenotype (e.g. *est1*Δ *upf2*Δ mutants senesce 23 to 28 PDs later than *est1*Δ). A plausible explanation would be that changes in mRNAs levels, caused by non-functional NMD, result in a decrease or an increase in the amount of certain proteins that effect the onset of senescence. When NMD response is disabled, the amount of several telomere related proteins as well as of RNAs is increased, for example the amount of telomerase subunits (Est1, Est2 and Est3), Ku complex, the Cdc13 partner proteins Stn1 and Ten1, and also Telomeric Repeat Containing RNA (TERRA) (Holstein et al., 2014).

1.5. NMD regulates the expression of CST complex subunits

In budding yeast proteins Cdc13, Stn1 and Ten1 form a trimeric complex (the so-called CST complex) that binds single stranded telomeric DNA. *CDC13*, *STN1* and *TEN1* are all essential genes and they are involved in the capping of telomeres. Since Stn1 and Ten1 bind ssDNA with lower affinity than Cdc13, it is considered that these proteins are recruited to telomeres via Cdc13. Except for its role in telomere end protection, CST is also known to interact with DNA polymerase alpha and this interaction is conserved (Holstein et al., 2014). The budding yeast CST complex still hasn't been purified but other data suggest that CST functions as a 2:4:2 or 2:6:2 complex (Lue et al., 2013). The three subunits of the complex show different functions, with

Cdc13 involved in recruitment of telomerase via interaction with Est1 and with Stn1 inhibiting telomerase activity by competing with Est1 for binding on Cdc13 (Holstein et al., 2014). It is also believed that Stn1 interacts with Pol12 and that, when overexpressed, it acts as an S phase checkpoint inhibitor. *TEN1* is also required for chromosome end protection and regulation of telomere length (Enomoto et al., 2014). As mentioned before, NMD pathway regulates the expression of *STN1* and *TEN1*, while expression of *CDC13* is not affected by this pathway. Furthermore, if NMD pathway is disabled, the ratio of CST components at telomeres will change (Figure 2). To prove this, Holstein and coworkers (2014) used a chromatin immunoprecipitation (ChiP) assay to measure binding of myc-tagged Stn1, Ten1 and Cdc13 to telomeric DNA in *nmd2*Δ background. They reported that binding of Stn1, Ten1 and Cdc13 to telomeres in *nmd2*Δ mutants increased by 10-fold, 5-fold and 2-fold respectively. Interestingly, the same phenotype was observed when *STN1* and *TEN1* were overexpressed as in the case of NMD deletion (Dahlseid et al., 2013). The latter can be explained with the rise in the level of Stn1 when NMD is not functional. Consequently, Stn1 negatively regulates telomerase by competing for binding on Cdc13 with Est1. As a result, telomeres are maintained at shorter length. In telomerase deficient *upf*Δ strains, the levels of Stn1 protein are also increased, which could be the reason why senescence is delayed in such mutants. One possible explanation is that excess Stn1 strengthens the telomere cap and chromosome ends are thus protected from degradation in telomerase-deficient cells (Addinall et al., 2011). Indeed, when Stn1 is overexpressed more of this protein is bound to telomeres independently of Cdc13 (Addinal et al., 2014). Since Stn1 has lower affinity for telomeric DNA than Cdc13, it is possible that Stn1 binds and caps the ends of telomeres by interacting with any of the other telomere binding proteins (e.g. Rap1, Rif1, Rif2, Ku, MRX, Tel1, telomerase, Sir proteins, RPA) or RNAs. The idea of Stn1 having physiologically important functions independently of Cdc13 is consistent with the data suggesting that the Ten1 interaction domain of Stn1 is much more critical for cell viability than the Cdc13 interaction domain (Petreaca et al., 2007).

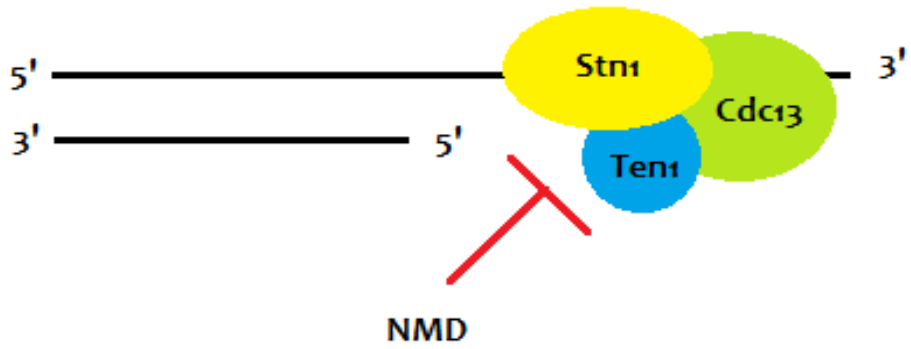

Figure 2. NMD controls the ratio of CST components at telomeres. Levels of mRNAs transcribed from *STN1* and *TEN1* are directly controlled by NMD. If NMD pathway is not functional, concentration of bound CST subunits at telomeres will increase, especially in the case of Stn1 that can bind telomeric DNA without Cdc13. (Figure courtesy of Paula van Mourik)

1.6. Aim of the study

This study aims to investigate the link between nonsense-mediated mRNA decay and type II survivor formation in *S. cerevisiae*. As mentioned before, Hu and coworkers (2013) reported that NMD genes - *UPF1*, *UPF2*, *UPF3* and *EBS1* are required for type II survivor formation in BY4741 yeast strain background. The first stage of my study will establish whether *UPF1*, *UPF2*, *UPF3* and *EBS1* are required for type II survivor formation in W303 yeast strain background and compare the made observations to the ones reported in Hu et al. (2013). In the second stage, I will focus on the exact link between NMD and survivor formation. As described in more detail in section 1.5, several observations that link NMD, CST complex and senescence have been reported. The fact that the overproduction of Stn1 results in the same senescing phenotype as has been observed for mutants with non-functional NMD, and the fact that binding of Stn1 to telomeres is increased in such mutants, suggest an additional function of Stn1, along with the ones it performs together with other subunits of the CST complex. It is possible that the presence of additional Stn1 at telomeres could intervene with recombination events that lead to survivor formation, explaining why a functional NMD pathway is required for type II survivor formation. These insights have prompted the idea to check whether Stn1 is the reason why NMD mutants are unable to form type II survivors.

2. Materials and methods

2.1. Yeast strains and media

All of the strains used in this study were *RAD5* derivatives of W303 (Thomas and Rothstein, 1989). In order to determine which NMD genes were required for type II survivor formation, NMD genes were individually deleted in the strain YPM9. This strain was previously created in Dr. Chang's laboratory and it harbored the gene deletions of *EST2* and *RAD51*. *EST2* codes for the catalytic subunit of telomerase and *RAD51* promotes strand invasion in homologous recombination and is necessary for type I survivor formation. Yeast cells in which *EST2* and *RAD51* are deleted cannot maintain and prolong their telomeres via telomerase or by recombination events leading to type I survivor formation. By deleting a certain gene in YPM9 strain and evaluating whether such cells can form type II survivors, it is possible to screen for genes important for type II survivor formation.

Next, to assess the potential function of *STN1* in blocking the formation of type II survivors, *STN1* was overexpressed in YPM9 strain. Along with YPM9, CCY6 and CCY7 strains were also used in the study. In these strains, *RAD51* was overexpressed in order to see whether this would affect type II survivor formation. CCY6 harbors a deletion of *EST2*, while CCY7 strain is characterized by the deletion of *TLC1* that encodes the RNA template component of telomerase.

Listed in Table 1 are all the strains that were used in this study.

Table 1. Strains constructed in order to verify the existence and establish the nature of the link between NMD and type II survivor formation.

Strain	**Genotype**	**Source**
YPM9	*MAT**a**/α est2::URA3/EST2 rad51::natMX4/RAD51*	Laboratory collection
CCY6	*MAT**a**/α est2::URA3/EST2*	Laboratory collection
CCY7	*MAT**a**/α tlc1::HIS3/TLC1*	Laboratory collection
AKY1	*MAT**a**/α est2::URA3/EST2 rad51::natMX4/RAD51 upf1::kanMX4/UPF1*	This study
AKY2	*MAT**a**/α est2::URA3/EST2 rad51::natMX4/RAD51 upf3::kanMX4/UPF3*	This study
AKY3	*MAT**a**/α est2::URA3/EST2 rad51::natMX4/RAD51* CUpr-STN1*/STN1*	This study
AKY4	*MAT**a**/α est2::URA3/EST2 rad51::natMX4/RAD51* GALpr-STN1*/STN1*	This study
AKY5	*MAT**a**/α est2::URA3/EST2* GALpr-RAD51*/RAD51*	This study
AKY6	*MAT**a**/α tlc1::HIS3/TLC1* GALpr-RAD51*/RAD51*	This study
AKY7	*MAT**a**/α est2::URA3/EST2 rad51::natMX4/RAD51 upf2::kanMX4/UPF2*	This study
AKY8	*MAT**a**/α est2::URA3/EST2 rad51::natMX4/RAD51 ebs1::kanMX4/EBS1*	This study

Three growth media were used while working with the described strains – yeast extract-peptone-dextrose (YPD), yeast extract-peptone-dextrose with added $CuSO_4$ (YPD+Cu) and yeast extract-peptone-galactose (YPGal). YPD and YPGal were used for single-colony streaking assays and liquid senescence assays. YPD+Cu was used only for the single-colony streaking assay. The media were prepared according to the recipe in Appendix I.

2.2. LiAC transformation

YPM9, CCY6 and CCY7 strains were transformed by lithium acetate (LiAc) method, where LiAc was used to permeabilize the cell wall of yeast cells. Overnight (O/N) cultures were grown in 5 ml YPD medium in rotating wheel in the incubator at 30^{o}C. The following day, cell density of the O/N culture was measured with a spectrophotometer. Based on the measured OD_{600}, a new culture was grown to mid log-phase (OD_{600}=0.4 to 0.8). These cells were then pelleted, washed with dH_2O, pelleted again and then resuspended in 1xTE/LiAc buffer. 50µL of the prepared yeast suspension was transferred to a new 1.5 ml Eppendorf tube together with 10-15 µL DNA (PCR product) and 5-10 µL ssDNA (50-100 µg was used for 1 transformation). 400 µL of 40%

PEG400 solution was added to the previously prepared transformation mixture. The tubes were incubated for 30 minutes at 30^{o}C and heat shocked in a water bath at 42^{o}C for 40-45 minutes. The cells were spinned down, resuspended in 200 µl YPD medium and let to grow for 1-2 hours in the incubator at 30^{o}C. 200 µL of the cell culture was plated on YPD plates, which were then incubated for 1 day at 30^{o}C. Finally, the colonies were replica plated on appropriate selection plates to select for the strains in which construct was integrated in the genome. The plates were incubated for 1-2 days at 30^{o}C

Solutions used for strain transformation are listed in Appendix II.

2.3. Polymerase-chain reaction (PCR)

UPF1, *UPF2*, *UPF3* and *EBS1* deletion mutants were identified in Yeast Knock-Out MAT*a* collection. This collection contains mutants in which a certain gene is deleted and replaced with the insertion of the kanamycin-resistance marker (*kanMX*) cassette. Genomic DNA (gDNA) was isolated from the mentioned mutants according to the protocol in Wizard® Genomic DNA Purification Kit (Promega). PCR was used to obtain the gene deletions containing a kanamycin-resistance marker that confers resistance to the antibiotic geneticin. PCR was set up according to the protocol from the Phusion High-Fidelity DNA Polymerase (Biolabs) manual. For every gene of interest, a pair of primers that bind 300-500 bp upstream and downstream of the gene was constructed (Table 2).

Table 2. Primers used for PCR reactions

Primer	Primer sequence	Description
UPF1_f	5'-TTAATGAATTCATGAACGGGA-3'	Primers bind 420 bp and 300 bp upstream and downstream of the *UPF1* respectively
UPF1_r	5'-CTTTATTACGCATTGCAGTGC-3'	
NMD2_f	5'-TGATACGAATTGATGGAGCCTG-3'	Primers bind 300-400 bp upstream and downstream of *UPF2*
NMD2_r	5'-TACAGCGGTGGTAAAGAAGAC-3'	
UPF3_f	5'-GGATTTTATTGCCGTCTTTTT-3'	Primers bind 420 bp upstream and downstream of *UPF3*
UPF3_r	5'-TTTATTTAGTCGGGGTTGTGG-3'	
EBS1_f	5'-CGGGAAATATCACAATGGAAG-3'	Primers bind 430 bp upstream and downstream of *EBS1*
EBS1_r	5'-TGACGCGTTCAAACCAGATT-3'	
kanB_r	5'-CTGCAGCGAGGAGCCGTAAT-3'	Binds onto *kanMX* cassette
STN1_S1	5'-GGGACAGAGGACGTTCGAATATTTGTTGGAGA TTTAAAGTACCGGATGCGTACGCTGCAGGTCGAC-3'	3'-end sequence of the primers enables binding to the pymN-1 and pymN-22, while 5'-end sequence is homologues to the region upstream and downstream of *STN1*
STN1_S4	5'-ATAACAAACATCGCCTTCTTGATGAGCTATAT GTCCGTACTTATCCATCGATGAATTCTCTGTCG-3'	
RAD51_S1	5'-CGTAGTTATTTGTTAAAGGCCTACTAATTTG TTATCGTCATATGCGTACGCTGCAGGTCGAC-3'	3'-end sequence of the primers enables binding to the pymN-1 and pymN-22, while 5'-end sequence is homologues to the region upstream and downstream of *RAD51*
RAD51_S4	5'-CCGTACTGAAGCTGTGACTCTGATATATGTTT TCTTGAACTTGAGACATCGATGAATTCTCTGTCG-3'	
STN1_f	5'-ATGGGCAAAGTCACTCAGAA-3'	Primers bind 200bp upstream and downstream of STN1
STN1_r	5'-AAAGCGACGGTTTTTGTCTT-3'	
RAD51_f	5'-TGGACGGTAAATGTTGGA-3'	Primers bind 200bp upstream and downstream of RAD51
RAD51_r	5'-CGTCGAAACGAAGACAAG-3'	

Sequences of *UPF1*, *UPF2*, *UPF3* and *EBS1* were taken from the *Saccharomyces* Genome Database (SGD).

In all of the PCR reactions in this study, Phusion® High-Fidelity DNA Polymerase (BioLab inc.) for amplification of DNA and 5X Phusion HF Buffer were used. The substances used and their final concentration are listed in Table 3 below. Table 4 contains parameter values for each step in

the PCR reaction. Gel electrophoresis was carried out in a horizontal tank containing 1xTE buffer and was run at 120 V for 30 minutes.

After the transformation of YPM9 strain, integration of the gene deletions was checked with PCR. Reaction mixture was set up according to the Table 3, the only difference being in the reverse primer used (in this case *kanMX* primer KanB). Except for the annealing temperature (T_a) and extension time, PCR conditions were the same as the ones specified in Table 4. T_a was set to 56^{o}C in the case of *UPF1*, *EBS1*, *UPF2* and to 55^{o}C in the case of *UPF3* gene deletions. Extension time was 30 seconds (for *UPF1*), 45 seconds (for *EBS1*) and 60 seconds (for *UPF2* and *UPF3*).

Table 3. PCR reaction mixture used to amplify *UPF1*, *UPF2*, *UPF3* and *EBS1* deletions

Component	**50 µl reaction**	**Final concentration**
5x HF buffer	10 µl	1x
Primer FW	2.5 µl	10 µM
Primer RV	2.5 µl	10 µM
dNTPs	1 µl	10 mM
Phusion DNA polymerase	0.5 µl	1.0 unit/50 µl PCR
gDNA	1 µl	50-250 ng/µL
Nuclease- Free Water	32.5 µl	

Table 4. PCR conditions for amplification of the *UPF1*, *UPF2*, *UPF3* and *EBS1* deletions

Step	**Temp ^{o}C**	**Time**	**Cycles**
Initial denaturation	98	30 sec	1
Denaturation	98	10 sec	30-40
Annealing	56	30 sec	30-40
Extension	72	*	30-40
Final extension	72	5 min	1
Hold	4	∞	

* extension time was 70 seconds in the case of *UPF1*, *UPF2*, *UPF3* and 45 seconds in the case of *EBS1*

2.4. Strain construction for *STN1* overexpression

In order to overexpress *STN1* in YPM9 strain the strategy described in Janke et al. (2004) was followed. The strategy requires: (a) a pair of primers that contain sequences of homology to the genomic target location within their 5' region; and (b) PCR-cassettes (also termed 'modules') that can be amplified using these primers. PCR-cassettes are located on different plasmids and contain a specific combination of promoters, selection markers and tags. There are many different modules so one can choose between them and select the one that carries the desired promotor, marker and/or tag. The strategy for overexpression of *STN1* is based on integration of a specific promotor in front of the gene. Two promotors were used in this study, galactose promotor (GALpr) and copper promotor (CUpr). Janke and associates (2004) named plasmids that carry these promotors pymN-1 (contains CUpr) and pymN-22 (contains GALpr). Except for promotors, the PCR modules of pymN-1 and pymN-22 also carry *kanMX4* cassette. According to the strategy described in the article, primers S1 and S4 should be used with pymN-1 and pymN-22 to successfully amplify CUpr/GALpr and *kanMX4* cassette (Figure 3). Bearing in mind that in the end CUpr/GALpr and *kanMX4* cassette should be integrated in front of *STN1*, primers that were identical to S1 and S4 primers were constructed, except for the additional 5' nucleotide sequence that was homologous to the genomic nucleotide sequence upstream of the *STN1* (Figure 4). The sequence of each primer is shown in Table 2. Because these primers are identical in sequence to S1 and S4 primers, they bind to pymN-1 and pymN-22 module and amplify the desired promoter and marker.

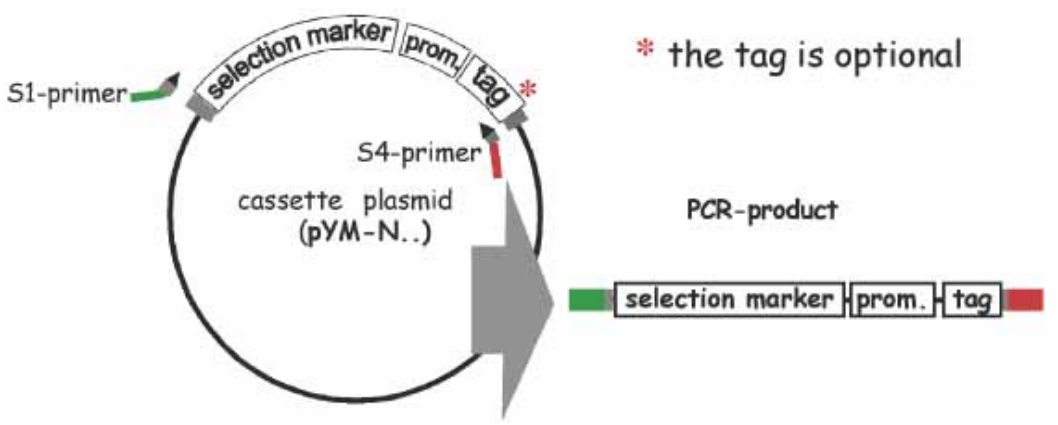

Figure 3. Amplification of PCR cassette. Each plasmid contains a PCR cassette, which consists of selection marker, promoter sequences and/or sequences that encode for a tag. From a diverse collection of such plasmids described in Janke et al. (2004), pymN-1 and pymN-22 was used. Their PCR cassettes both contain kanamycin resistance marker, along with CUpr (pymN-1) or GALpr (pymN-22), while none of them contains any sequence that encodes for a tag. S1- and S4-primers allow amplification of cassettes of pymN-1 and pymN-22. (Adapted from Janke et al., 2004)

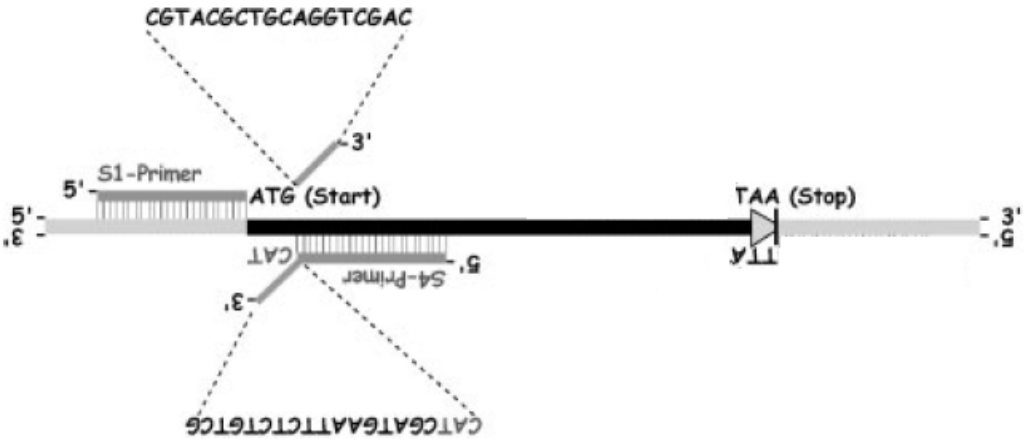

Figure 4. Primer design (Janke et al., 2004). The 5’ end of S1-primer consists of 45–55 bases that are homologous to the sequence upstream of the ATG (including ATG = start codon) of *STN1*. The 3’ end contains the 5-CGTACGCTGCAGGTCGAC-3 sequence, which enables binding of the primer to appropriate PCR modules. The 5’ end of S4-primer is the reverse complement of 45–55 bases downstream of the ATG (start-codon) of *STN1* (excluding ATG) and the 3’ end consists of the 5-CATCGATGAATTCTCTGTCG-3 sequence, crucial for binding to appropriate PCR modules.

PCR cassettes of pymN-1 and pymN-22 were amplified according to the protocol for hot-start PCR that is used in Dr. Chang's laboratory. The components used and their final concentrations are shown in Tables 5 and 6 below. Steps of the PCR program are listed and described in Table 7. After PCR, amplicons were transformed in YPM9 strain. The constructs were integrated in front of *STN1*, enabling overexpression of *STN1* by addition of galactose or copper to the media.

The same procedure was performed in the case of CCY6 and CCY7 strains, in which *RAD51* using GALpr was overexpressed. Used primers are presented in Table 2.

Integration of the promoter at the specific location in the genome was later checked by PCR. Reaction mixture was set up according to Table 3, the only difference being the primers used. For proving the integration of PCR cassette in front of *STN1*, primers STN1_f and STN1_r were constructed (Table 2). In the case of *RAD51*, primers RAD51_f and RAD51_r were used (Table 2). Except for the annealing temperature (T_a) and extension time, PCR conditions were the same as the ones specified in Table 4. For proving the integration of PCR cassette in front of *STN1* and *RAD51*, T_a and extension time were set to 55^{o}C and 60 seconds respectively.

Table 5 PCR mix 1 for amplification of pymN-1 and pymN-22 PCR cassettes

Mix 1		
Component	40 µl reaction	Final concentration
5x HF buffer	8 µl	1x
Primer FW	1 µl	10 µM
Primer RV	1 µl	10 µM
dNTPs	1 µl	10 mM
DNA (plasmid)	1 µl	10-20 ng/40µl
Nuclease-Free Water	28 µl	

Table 6 PCR mix 2 for amplification of pymN-1 and pymN-22 PCR cassettes

Mix 2		
Substances	10 µl reaction	Final concentration
5x HF buffer	2 µl	1x
Phusion DNA polymerase	0.25 µl	0.5 units/10 µl
Nuclease-Free Water	7.75 µl	

Table 7 Hot-start PCR program

PCR mix	Step	Temp ^{o}C	Time	Cycles
Mix1	Initial denaturation	95	5 min	∞
Add mix 2	Denaturation	95	30 sec	30
	Annealing	68	30 sec	30
	Extension	72	30 sec	30
	Final extension	72	10 min	1
	Hold	4	∞	

2.5. Tetrad dissection and mutant selection

After evaluating the success of the transformations described in 2.1., the same procedure was followed each time: the transformants were streaked on minSPO plates (see Appendix I) and incubated at 22^{o}C for 7-10 days. Over that period of time, yeast cells underwent sporulation. Cells were taken from each plate and were put into an Eppendorf tube that already contained zymolyase solution (concentration 0.5 mg/ml). The tubes were incubated for 15 minutes at room temperature (RT). After the lysis of yeast cell walls by zymolyase, solution from one tube was spread on YPD plate and further analyzed with dissection microscope. Separation of ascospores from individual asci was carried out with a glass microneedle attached to the micromanipulator on the dissection microscope. Ascospores were organized in 12 rows on a single YPD plate, each row containing four ascospores from one ascus. In the case of AKY3 and AKY4 strain, tetrad dissection was performed both on YPD and YPD+Cu or YPGal plates respectively. After 3-4 day incubation at 30^{o}C, newly formed colonies were replica plated on selection plates. The tetrads plates from strains AKY1-4, AKY7 and AKY8 were replica plated on YPD+G418, YPD+NAT and SD-URA plates (for ingredients see Appendix I). In the case of AKY5 and AKY6 strains, colonies on each YPD plate were replica plated on YPD+G418, SD-URA and SD-HIS plates.

After 24-hour incubation, 2:2 segregation of the markers linked to the gene deletion was observed. In the case of AKY1-4, AKY7 and AKY8 strains, single (*est2*ΔURA3), double (*est2*ΔURA3 *rad51*Δ*natMX4*, *est2*ΔURA3 xxxΔ*kanMX4* and *rad51*Δ*natMX4*xxxΔ*kanMX4*) and triple (*est2*ΔURA3 *rad51*Δ*natMX4* xxxΔ*kanMX4*) mutants were identified. With AKY5 and AKY6 strains single (*est2*ΔURA3, *tlc1*ΔHIS3, GALpr-*RAD51*) and double (*est2*ΔURA3 GALpr-*rad51*, *tlc1*ΔHIS3 GALpr-*RAD51*) mutants were observed.

2.6. Single-colony streaking assay

After tetrad dissection and identification of the mutants listed in the previous paragraph, a single-colony streaking assay was performed to determine whether mutants are able to form type II survivors. Single, double and triple mutants were streaked on separate YPD plates, divided into 6 sections. The mutants were initially streaked only on the first section of each plate, after which

the plates were incubated for 3 days at 30^{o}C. During the incubation new colonies formed and one of them was taken from the first section and streaked on the second section of the same plate. Plates were then again incubated for 3 days at 30^{o}C. The described streaking procedure was repeated 5 times in total. During the procedure, mutants started senescing and afterwards formed type II survivors or died.

To test the effect of overexpressing *STN1* or *RAD51*, strains AKY3, AKY4, AKY5 and AKY6 were streaked on both YPD and YPD+Cu or YPGal plates as described above.

2.7. Liquid senescence assay

After tetrad dissection and identification of mutants, colonies of single, double and triple mutants were inoculated O/N in 2.5 ml of the appropriate liquid medium at 30^{o}C. The next day, which represented day 0 time point, the cell density of the O/N culture was measured using CASY cell counter (Roche Life Science). Since cell density of the O/N cultures was too high to measure directly, dilutions described below were prepared. 100 µl of the O/N culture was diluted in 900µl dH_2O in a 1.5 ml Eppendorf tube. 10µl of the solution was then added to 10 ml of CASY buffer. Cell density was measured in order to calculate the exact volumes of O/N cultures that had to be added to 5 ml of YPD or YPGal medium for the final concentration of cells in every new tube to be 2x10^{5} cells/ml. Cultures were then grown for 24 hours at 30^{o}C. The following day, which represented day 1 time point, the procedure described above was repeated - cell density was measured and new cultures with identical starting concentration of yeast cells were prepared. These cultures were once again grown for 24 hours. The same procedure was repeated for the next ten days. Every day, cells were taken for Southern blot analysis.

In this assay, cell density of every culture was measured over 10 days. Telomerase-deficient yeast cells were dividing in the liquid culture until they reached senescence, after which the rate of culture growth started decreasing. However, upon further incubation, cell density started increasing again because the telomeres were elongated by recombination-based telomere maintenance mechanism (Figure 5). The main objective of this assay was to determine the growth phenotype of the mutants and determine whether single, double and triple mutants would be able to form survivor cells.

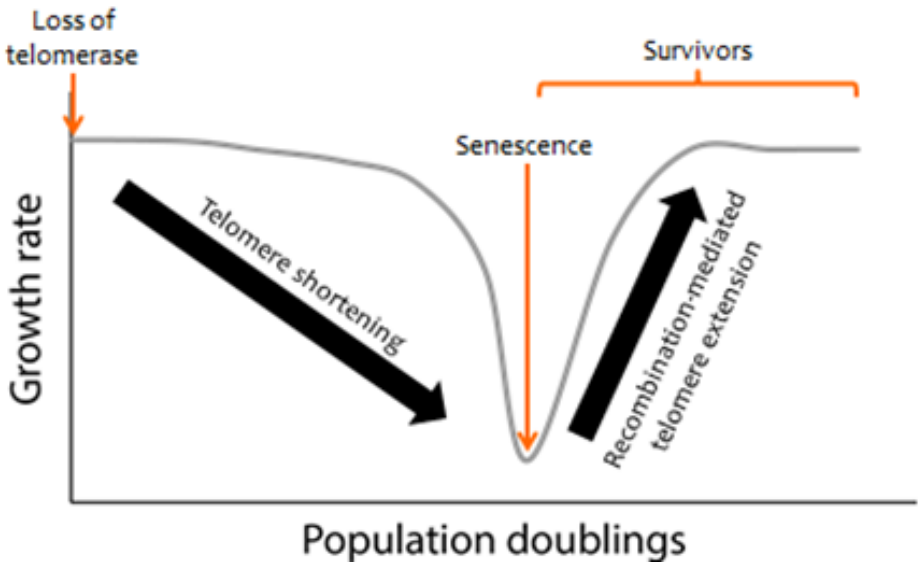

Figure 5. Events influencing growth rate changes during liquid senescence assay. In telomerase-deficient yeast cells, telomeres shorten progressively. When the telomeres reach their critical length, cells enter a state that blocks cell division, termed senescence. As a result, growth rate will progressively decrease since the number of cells that have entered senescence is increasing. Some cells will bypass the senescence by prolonging their telomeres through recombination-mediated events, giving rise to survivors.

2.8. Telomere Southern blot

Genomic DNA was isolated from samples collected during liquid senescence assay using Wizard® Genomic DNA Purification Kit (Promega). Isolated gDNA was digested overnight with *Xho*I at 37°C and separated on 1% agarose gel with EtBr that was run for 10 minutes at 100 V and then for 2-3 hours at 120 V. DNA was depurinated by incubation of the gel in 0.25 M HCL for 15 minutes followed by denaturation of the gel for 30 minutes in 0.4 M NaOH. DNA was transferred to a Hybond-N^+ membrane (GE Healthcare) by vacuum transfer method (5 Hg for 60 minutes in 10xSSC buffer). Afterwards, the membrane was rocked for 1 hour in denaturation solution and later on for 2x10 minutes in neutralization solution at RT. The membrane was then pre-hybridized in hybridization incubator in 20 ml pre-warmed DIG easy hyb buffer (Roche) at 39°C for 1 hour. The membrane was probed with TG_{1-3} telomere-specific probe labeled with digoxigenin (DIG, probe was labelled according to the DIG oligonucleotide 3'-end labelling kit, 2nd generation Roche). Hybridization happened overnight at 39°C. Next day, the membrane was washed 2x5 minutes with pre-warmed 2xSSC + 0.1% SDS and then 2x20 minutes with pre-

warmed 0.5xSSC + 0.1% SDS at 39^{o}C. After the washing steps, the membrane was briefly rinsed in 25 ml of 1x DIG wash buffer and then blocked for 30 minutes with 10 ml of 1x blocking solution at RT. The probe was marked with AP-coupled anti-DIG Fab (Roche) that was diluted in 10 ml 1x blocking solution (1:10000 dilution) and membrane incubation lasted for 30 minutes at RT. In the end, 1 ml of solution of DIG detection buffer with CSPD (100x diluted) was spread over the membrane, which was incubated for 5 minutes in the dark and then for 15 minutes at 37^{o}C, the latter ensuring that the signal would be enhanced. Samples were visualized by using the Chemidoc system.

Solutions that were prepared for Southern blot are listed in Appendix III. After preparation, the solutions were autoclaved and stored at RT.

3. Results

3.1. Assessing the outcome of AKY1-AKY8 strain creation

As mentioned in section 2.1., strains AKY1-AKY8 were created by transforming YPM9, CCY6 and CCY7 strains with the appropriate construct. This section describes the results which confirm the success of the transformation and prove the accuracy of construct integration at the specific position in the genome.

After genomic DNA (gDNA) of *UPF1*, *UPF2*, *UPF3* and *EBS1* deletion mutants was isolated and the respective gene deletions containing a *kanMX* marker were amplified, the PCR products were transformed into YPM9. The specificity of the integration of the products was checked with PCR (Figure 6). Forward primers that bind ~400 bp upstream of the deleted genes and a reverse primer that binds to the *kanMX* cassette were used. In order for the transformation to be deemed successful and specific, the primers had to amplify the region of around 1800 bp in each reaction. Amplicons thus had to contain almost whole *kanMX* cassette sequence which is 1506 bp long and a ~400 bp long sequence upstream of the target gene deletion. PCR products were analysed on 1% agarose gel and stained with ethidium bromide (EtBr). With all the transformants, the obtained PCR products were around 1800 bp long, which proved the efficient construction of AKY1, AKY2, AKY7 and AKY8 strains.

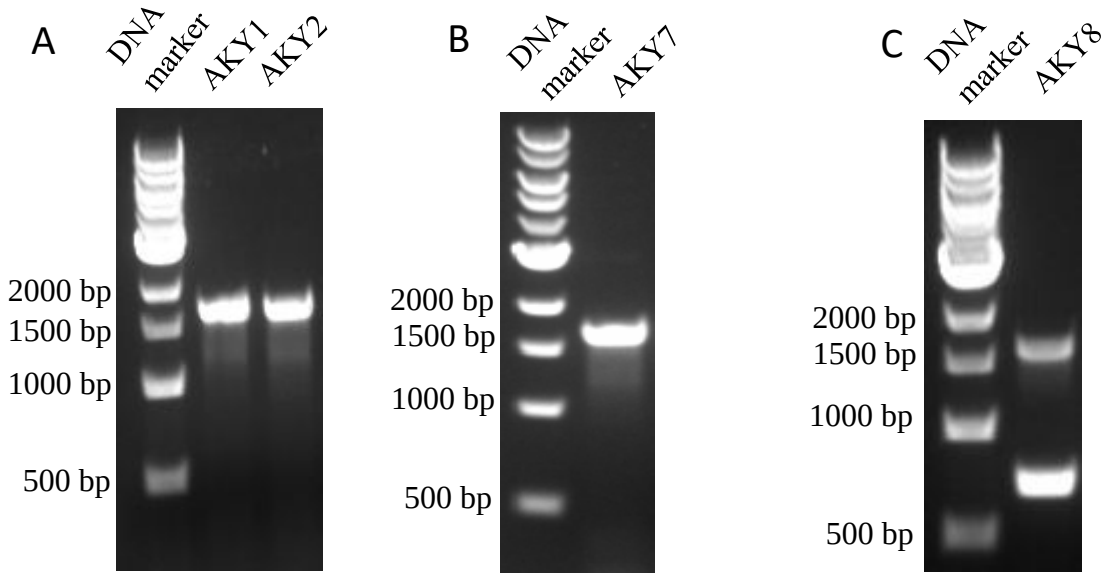

Figure 6. PCRs performed to evaluate AKY1/AKY2 (A), AKY7 (B) and AKY8 (C) strain construction. PCR products (~1800 bp long) representing *UPF1*/*UPF3*, *UPF2* and *EBS1* deletions are seen on the gels. Strong band representing the ~700 bp fragment (C) is caused by unspecific amplification.

The strategy for the overexpression of *STN1* and *RAD51* included integration of pymN-1 and pymN-22 PCR cassettes upstream of *STN1* or *RAD51*. The length of the pymN-1 and pymN-22 PCR cassettes was 1990 bp and 1978 bp respectively. As described in section 2.3., a specific pair of primers whose 3' ends enabled binding to the appropriate PCR modules and whose 5' ends were homologous to the region upstream and downstream of *STN1* or *RAD51* was constructed. Firstly, these primers were used for amplification of PCR cassettes of pymN-1 and pymN-22. Secondly, the obtained amplicons were transformed into YPM9 or CCY6 and CCY7 strain. Accuracy of the integration of the cassettes was checked with PCR. Primers were constructed so that the forward primer bound ~200 bp upstream of the start codon while the reverse primer bound ~200 bp downstream of the stop codon of either *STN1* or *RAD51*. Hence, if the integration of PCR cassettes was specific, primers had to amplify the region that contained both the integrated PCR cassette and either *STN1* or *RAD51*. The length of PCR cassettes from pymN-1 and pymN-22 was 1990 bp and 1978 bp respectively. Since *STN1* is 1485 bp long, the PCR products in total had to be 3828 bp (for pymN-1 cassette) and 3816 bp (for pymN-22 cassette); in the case of 1203 bp long *RAD51*, the PCR product had to be 3581 bp (for pymN-22 cassette). Nevertheless, since the analyzed transformants were diploids, a PCR cassette was integrated only

in front of one copy of the gene of interest. Because of that, it was also expected that the primers would amplify only *STN1* or *RAD51* so that additional bands ~400 bp longer than the two genes would be seen on the gel. The PCR products were analyzed on 1% agarose gel and stained with EtBr. Pictures of gels are shown in Figure 7. The PCR products that suggest precise integration of PCR cassettes are marked in green. The bands with the strongest signal represent *STN1* and *RAD51* upstream of which integration of PCR cassettes did not occur. Other bands are a consequence of non-specific binding of the primers.

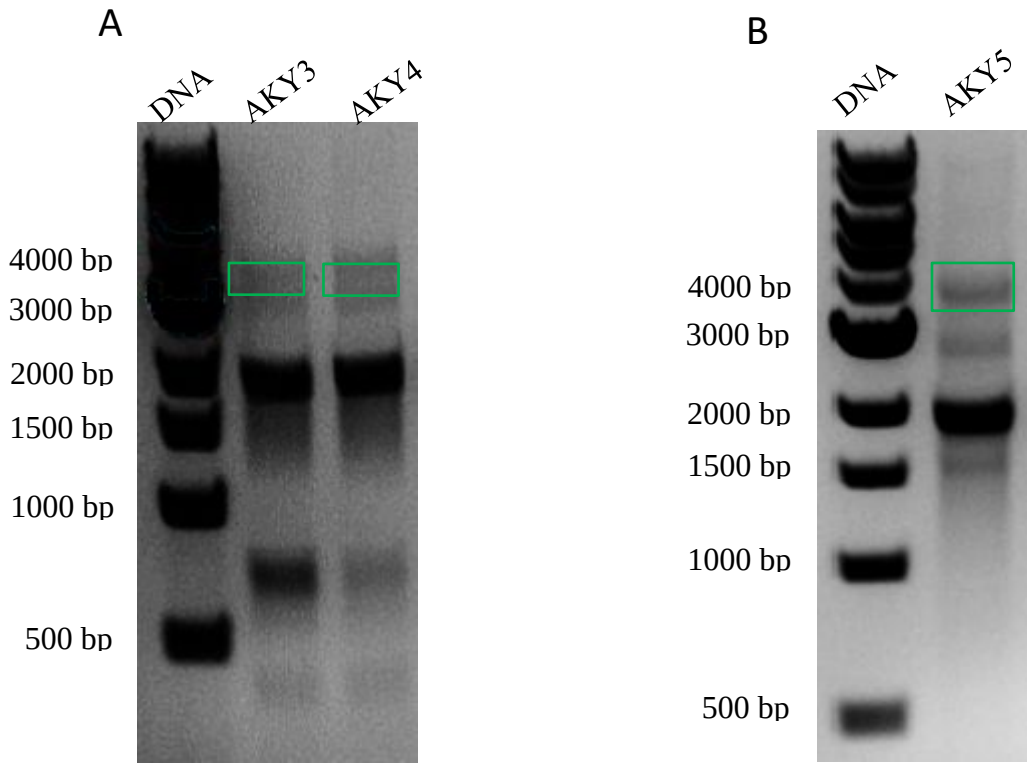

Figure 7. (A) PCR performed to prove the integration of PCR cassettes from pymN-1 and pymN-22 in front of *STN1*. A fragment of the expected size, 3828 bp for pymN-1 cassette and 3816 bp for pymN-22 cassette, is seen on the gel for each of the two strains (AKY3 and AKY4). The bands representing these fragments are marked in green. (B) PCR performed to prove the integration of PCR cassette from pymN-22 in front of *RAD51*. The band representing the fragment of the expected size (~3581 bp) is marked in green.

3.2. Importance of *UPF1* and *UPF3* for type II survivor formation

In order to investigate whether type II survivor formation depends on *UPF1* and *UPF3*, I created knock-out mutants of the mentioned genes and observed if they would be able to form type II survivors. After the sporulation of AKY1 and AKY2, tetrad dissection was performed. Based on the ability of cells to form colonies on YPD+G418, YPD+NAT and SD-URA plates, single, double and triple mutants were identified. The mutants were then streaked on YPD plates and single-colony streaking assay was performed. For each strain I streaked 10 single, 20 double and 20 triple mutants. All of them were telomerase-deficient, which meant that yeast cells were able to divide only a certain number of times before they started senescing. As expected, a small number of cells formed survivors and thus bypassed senescence. When the number of colonies started rising, the new colonies were most likely formed from survivor cells. In the case of telomerase-deficient mutants with *RAD51* deleted, it is believed that such colonies were formed from type II survivors. In other instances cells senesced and no new colonies formed in later sections of the plate. Such an outcome in case of triple mutants indicated that the deleted gene was somehow involved in type II survivor formation. At the end of the streaking assay, I counted the plates on which survivors formed. The other plates contained mutants that senesced and were not able to form survivors. Such mutants were named dropouts (DOs). Percentage of DOs was calculated for single, double and triple mutants.

Results of single-colony streaking assay are shown in table 8 (for AKY 1 strain) and table 9 (for AKY2 strain). It can be seen from the tables that all of the single mutants (*est2*Δ) overcame senesce and that percentage of DOs therefore equaled 0. This was expected since single mutants were able to form both types of survivors.

Based on literature, double mutant *est2*Δ *rad51*Δ was expected to be unable to form type I survivors whose generation is dependent on *RAD51*. With regard to *est2*Δ *upf1*Δ or *est2*Δ *upf3*Δ mutants, if *UPF1* and *UPF3* were indeed required for type II survivor formation, only type I survivors were expected to form. Since all of the mentioned double mutants were able to form a certain type of survivors, no DOs were expected. Surprisingly, the percentage of DOs for *est2*Δ *rad51*Δ mutant from both AKY1 and AKY2 strain was noticeably high – 60% and 40% respectively. A plausible explanation of such an outcome had to do with the generally low

incidence of type II survivor formation and the possibility that survivor colonies were not picked and restreaked during single-colony streaking assay. This would be in line with the fact that a frequent occurrence of *est2*Δ *rad51*Δ DOs was also observed in other single-colony streaking assays performed earlier in the laboratory. As expected, no DOs of *est2*Δ *upf1*Δ or *est2*Δ *upf3*Δ mutants were observed.

Occurrence of DOs was most frequent in the case of triple mutants. This could indicate that both *UPF1* and *UPF3* are somehow required for type II survivor formation since deletion of these genes results in an increased number of DOs. Although the percentage of triple mutant DOs was high, the observed number of *est2*Δ *rad51*Δ DOs made it difficult to assess to what degree the deletion of *UPF1* or *UPF3* influenced type II survivor formation. This was particularly the case with AKY1 strain, where, in comparison to *est2*Δ *rad51*Δ mutants, deletion of *UPF1* only raised the percentage of DOs by additional 20%. Based solely on this result, it was difficult to claim that this increase in number of DOs was significant and sufficiently high to mean that *UPF1* deletion disturbed type II survivor formation. For AKY2 strain, the percentage of triple mutant DOs was 2 times higher than in the case of *est2*Δ *rad51*Δ mutant. In my opinion, this result could indicate that *UPF3* is required for type II survivor formation.

Table 8. Single-colony streaking assay performed with mutants of the indicated genotypes, derived from the sporulation of AKY1. The mutants were examined for the ability to bypass senescence by forming survivors. DOs - dropouts, i.e. mutants that senesced and were not able to form survivors; *est2*Δ - telomerase-deficient mutant; *est2*Δ *rad51*Δ - telomerase-deficient mutant with deleted *RAD51*; *est2*Δ *upf1*Δ - telomerase-deficient mutant with inactivated NMD pathway; *est2*Δ *rad51*Δ *upf1*Δ - telomerase-deficient mutant with deleted *RAD51* and inactivated NMD pathway.

Mutants	Number of DOs	% DOs
*est2*Δ	0/9 DOs	0
*est2*Δ *rad51*Δ	6/10 DOs	60
*est2*Δ *upf1*Δ	0/10 DOs	0
*est2*Δ *rad51*Δ *upf1*Δ	16/20 DOs	80

Table 9. Single-colony streaking assay performed with mutants of the indicated genotypes, derived from the sporulation of AKY2. The mutants were examined for the ability to bypass senescence by forming survivors. DOs - dropouts i.e. mutants that senesced and were not able to form survivors; *est2*Δ - telomerase-deficient mutant; *est2*Δ *rad51*Δ - telomerase-deficient mutant with deleted *RAD51*; *est2*Δ *upf3*Δ - telomerase-deficient mutant with inactivated NMD pathway; *est2*Δ *rad51*Δ *upf3*Δ - telomerase-deficient mutant with deleted *RAD51* and inactivated NMD pathway.

Mutants	Number of DOs	% DOs
*est2*Δ	0/10 DOs	0
*est2*Δ *rad51*Δ	4/10 DOs	40
*est2*Δ *upf3*Δ	0/10 DOs	0
*est2*Δ *rad51*Δ *upf3*Δ	17/20 DOs	85

3.3. Necessity of *UPF2* for type II survivor formation

Based on the results described in the previous section, it was logical to assume that *UPF2* might also be required for type II survivor formation. In their work Hu and coworkers (2013) claim that type II survivor formation in BY4741 yeast strain background depends on *UPF1*, *UPF2*, and *UPF3*. When results of single-colony streaking assay with AKY1 and AKY2 strains were gathered, liquid senescence assay was performed with AKY7 strain. This strain was tetrad dissected and mutants were identified, some of which were selected and used for liquid senescence assay. The assay was performed according to the instructions described in section 2.7.

Data acquired from the assay was processed and results were presented in the form of a graph, as shown in Figure 8. The x- and y-axes correspond to the number of population doublings (PDs) and to the cell density respectively. Cell density changes in the cultures of single (*est2*Δ), double (*est2*Δ *rad51*Δ and *est2*Δ *upf2*Δ), triple (*est2Δ rad51*Δ *upf2*Δ) mutants and two controls (WT and *upf2*Δ) were entered into the graph. Four cultures of each mutant and two cultures of each control were prepared and monitored during the assay. In the end, results for all the cultures of a given mutant or control were represented by a single function on the graph.

As it can be seen from Figure 8, the growth functions representing the controls did not show any significant fluctuations. In other words, there was no significant increase or decrease in the cell density, which means that WT and *upf2*Δ cells did not senesce but continued to grow in the cultures until they consumed all of the nutrients required for further growth. This was expected of both the controls, since telomerase was active. Functions representing *est2*Δ and *est2*Δ *rad51*Δ mutants started increasing with only a few PDs and later on they reached a plateau. Most likely, these mutants started senescing before the beginning of the liquid senescence assay so the senescing phenotype was not observed. Soon after the beginning of the assay, the cell density of these mutants started increasing due to survivor formation. The plateau phase was reached when a sufficient amount of nutrients was no longer available in the culture to initiate further cell growth. In the case of *est2*Δ *upf2*Δ mutants, cell density first started to slowly decrease and then continued to slowly increase. This mutant showed less severe senescing phenotype than was to be expected. Survivor formation happened later than in the case of the other mutants. As a result, the increase in cell density happened later in the assay. The most surprising result was the one for *est2*Δ *rad51*Δ *upf2*Δ mutant. This mutant showed a severely senescing phenotype and in the end cell density rose due to survivor formation. The type of the formed survivors was not verified with Southern blot but since these mutants should not be able to form type I survivors (due to *RAD51* deletion), a reasonable assumption was that the formed survivors were type II. This result contradicts the one reported by Hu and associates (2013), who claim that functional *UPF2* is required for type II survivor formation in BY4741 yeast strain background.

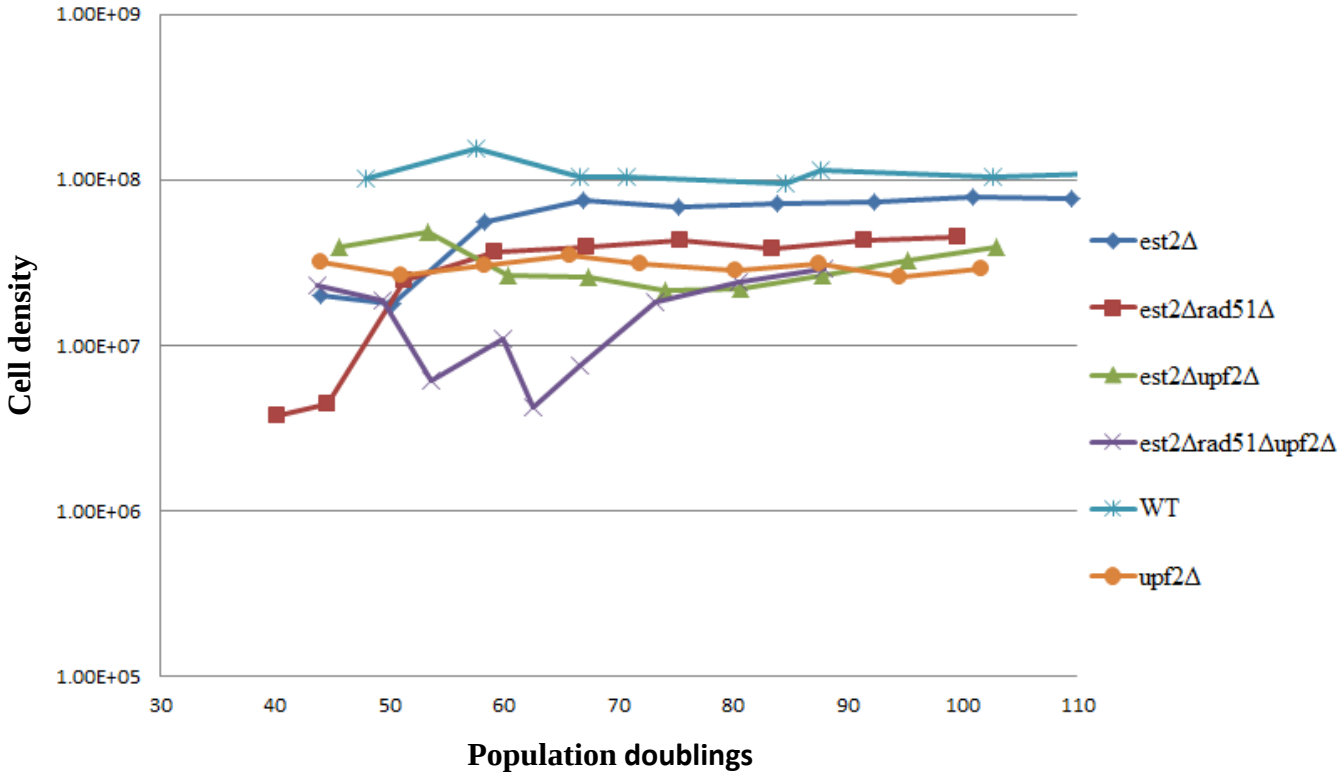

Figure 8. Type II survivor formation does not depend on *UPF2*. Growth rates were measured in liquid culture by serial passaging of haploid meiotic progeny of the indicated genotypes, derived from the sporulation of AKY7. *est2*Δ - telomerase-deficient mutant; *est2*Δ *rad51*Δ - telomerase-deficient mutant with deleted *RAD51*; *est2*Δ *upf1*Δ - telomerase-deficient mutant with inactivated NMD pathway; *est2*Δ *rad51*Δ *upf1*Δ - telomerase-deficient mutant with deleted *RAD51* and inactivated NMD pathway; WT - wild type; *upf2*Δ - mutant with inactivated NMD pathway.

3.4. The influence of *EBS1* deletion on the occurrence of type II survivors

Although the specific function of *EBS1* is still unknown, it is believed that it codes for a global inhibitor of translation. Ford and coworkers (2006) claim that *EBS1* inhibits the expression of many yeast genes, including ones controlling telomere length. Their results also suggest that overexpressing *EBS1* reduces gene expression without commensurate changes in transcript abundance, which distinguishes *EBS1* from *UPF* genes.

In addition to *UPF1* and *UPF3*, I tested whether *EBS1* is necessary for type II survivor formation. The reason for including *EBS1* in this study is its connection to NMD. Although *EBS1* is not required for NMD, the connection is evident in the fact that NMD factors interact with

translation machinery and in that the levels of *EBS1* mRNA are controlled by NMD pathway (Ford et al., 2006). Furthermore, it has been demonstrated that *EBS1* deletion leads to a slight but highly reproducible stabilization of NMD targets (Luke et al., 2007).

In order to assess the importance of *EBS1* for type II survivor formation, I constructed AKY8 strain. Following tetrad dissection, single, double and triple mutants were identified. Several mutant colonies were selected and used in single-colony streaking assay (10 different colonies in the case of single and double mutants and 20 in the case of triple mutant). When the assay was completed, the amount of mutants that senesced and did not form survivors was determined (Table 10). As expected, no *est2*Δ DOs were observed. On the other hand, as was the case with AKY1 and AKY2 strain, the percentage of *est2*Δ *rad51*Δ DOs was 40% and thereby higher than was expected. *est2*Δ *ebs1*Δ mutants formed survivors on almost all of the plates, except for one mutant. All but the one mutant most likely formed type I survivors since this survivor type is generally found more frequently on solid-medium-grown cultures than type II. Nevertheless, since it seems that deletion of *EBS1* does not completely block type II survivor formation, it is possible that type II survivors also formed on plates with *est2*Δ *ebs1*Δ mutants. In the case of triple mutant, the percentage of DOs was as high as 75%, which was almost twice as high as in the case of *est2*Δ *rad51*Δ mutant. This noticeable difference in the percentage of DOs indicates that *EBS1* might be involved in type II survivor formation.

Table 10. Single-colony streaking assay performed with mutants of the indicated genotype, derived from the sporulation of AKY8. The mutants were examined for the ability to bypass senescence by forming survivors. DOs - dropouts, i.e. mutants that senesced and were not able to form survivors; *est2*Δ - telomerase-deficient mutant; *est2*Δ *rad51*Δ - telomerase-deficient mutant with deleted *RAD51;* *est2*Δ *ebs1*Δ - telomerase-deficient mutant with deleted *EBS1; est2*Δ *rad51*Δ *ebs1*Δ - telomerase-deficient mutant with deleted *RAD51* and *EBS1*.

Mutants	Number of DOs	% DOs
*est2*Δ	0/10 DOs	0
*est2*Δ *rad51*Δ	4/10 DOs	40
*est2*Δ *ebs1*Δ	1/10 DOs	10
*est2*Δ *rad51*Δ *ebs1*Δ	15/20 DOs	75

3.5. Effects of *STN1* overexpression on type II survivor formation

3.5.1 Credibility of *STN1* overexpression with GALpr

Because GALpr was integrated in front of *STN1*, the expression of this gene depended on the presence of galactose. We wanted to examine how *STN1* regulation with GALpr would influence the growth phenotype of mutants which were grown with or without galactose. In order to do that, tetrad dissection was performed with AKY4 strain. WT and GALpr-*STN1* mutant were selected and used for single-colony streaking assay. They were streaked both on YPD and YPGal plates and later on the growth phenotype was examined. Because of regulation by GALpr, the expression of *STN1* had to be confined only to the cells growing on YPGal plates. With *STN1* being an essential gene, GALpr-*STN1* mutants could not be expected to grow on YPD plates when Stn1 was not produced. Surprisingly, as can be seen from the plates presented in Figure 9, many colonies of this mutant formed on YPD plates. Although GALpr-*STN1* colonies were less abundant than WT colonies on YPD plate, they were still present in high numbers. The most probable explanation for this observation is that GALpr was leaky so that a certain amount of Stn1 was translated. Since *STN1* was expressed on YPGal plates GALpr-*STN1* mutants were able to grow no worse than WT on those plates. This can be seen from Figure 9, which shows that the density of WT and GALpr-*STN1* colonies grown on YPGal was similar and that WT cells grew equally well on YPD and YPGal plates.

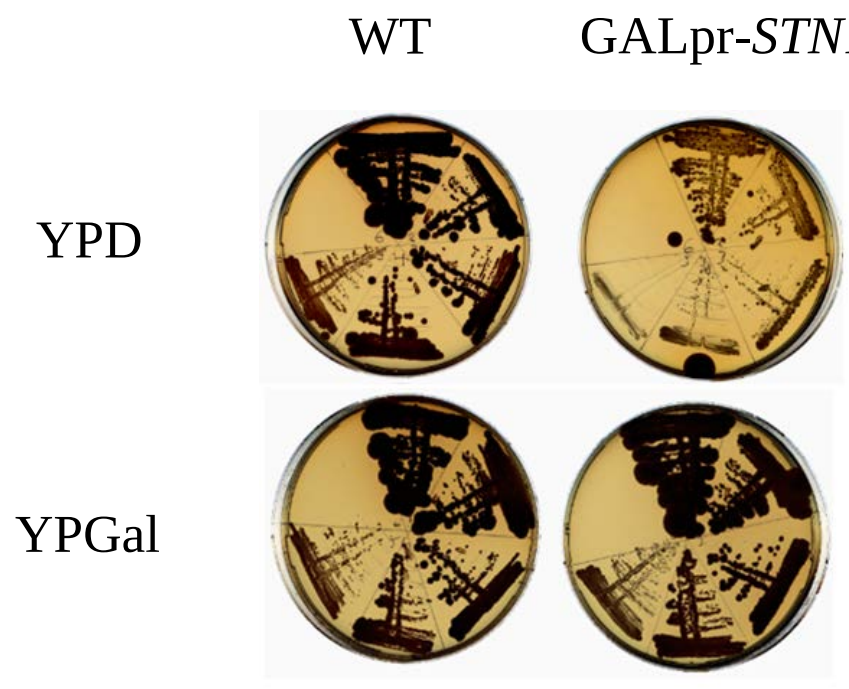

Figure 9. Single-colony streaking assay performed with WT and GALpr-*STN1* mutant. Growth phenotype of GALpr-*STN1* mutant grown on YPD plate differs from the other three. The fact that some colonies have grown indicates that GALpr is leaky. WT - wild type; GALpr-*STN1* - mutant with *STN1* expression controlled by GALpr.

3.5.2 Single-colony streaking assay with AKY3 and AKY4

Several assays were performed in order to determine whether the rise in the concentration of Stn1 would result in an inability of yeast cells to form type II survivors. One of them was single-colony streaking assay performed with AKY3 and AKY4. In these strains the expression of *STN1* was controlled with CUpr and GALpr respectively. As described in section 2.6., single, double and triple mutants of each strain were streaked both on YPD and either YPD+Cu or YPGal plates. In this way, the growth phenotype of mutants with and without *STN1* overexpression was monitored. When the assay was completed, DOs of each mutant were counted. Genotypes of the streaked mutants and percentage of DOs are shown in Table 11 (for AKY4) and Table 12 (for AKY3).

With respect to both strains the obtained results were not completely clear. With AKY4 strain, single mutants were able to form survivors and no DOs were present, just as it had been expected. Regarding *est2*Δ *rad51*Δ mutants, a high percentage of DOs was observed, just as was

the case in the single-colony streaking assays described in 3.2. and 3.4. It may have happened that random colonies with no survivor cells were picked, which would account for the high number of DOs. Results were even more surprising in the case of *est2*Δ GALpr-*STN1* mutant. In accordance with the generally held belief, this mutant was expected to form type I survivor cells. In case that type II survivors would also be formed, the hypothesis about NMD regulating type II survivor formation through *STN1* would not be confirmed. Contrary to the expectation about survivor formation, the percentage of DOs on YPGal plates was as high as 80%. The observed result might suggest that *STN1* blocks both type I and type II survivor formation. The percentage of DOs on YPD plates was 60 %. With *STN1* being an essential gene, *est2*Δ GALpr-*STN1* mutant should not have been able to grow on YPD plates since *STN1* was not expressed. Also unexpectedly no DOs of *rad51*Δ GALpr-*STN1* mutant were observed on YPD plates, most likely due to the leakiness of GALpr. Results for triple mutants indicated that upregulation of *STN1* expression resulted in the inability of cells to form type II survivors. The main argument for this was the percentage of DOs, which was higher for the mutants grown on YPGal than for the mutants grown on YPD. Nevertheless, other results, especially the one for *est2*Δ GALpr-*STN1* mutant, did not support this claim. It was puzzling that the percentage of *est2*Δ GALpr-*STN1* DOs (80%) was higher than the one for triple mutants (75%). Even if there is no connection between *STN1* expression and type II survivor formation, fewer DOs should have been observed in the case of *est2*Δ GALpr-*STN1* mutant, since this mutant should generally be able to form type I survivors.

Table 11. Single-colony streaking assay performed with mutants of the indicated genotype, derived from the sporulation of AKY4. Telomerase-deficient mutants were examined for the ability to bypass senescence by forming survivors. *rad51*Δ GALpr-*STN1* mutant with functional telomerase is used as a control. DOs - dropouts, i.e. mutants that senesced and were not able to form survivors; *est2*Δ - telomerase-deficient mutant; *est2*Δ *rad51*Δ - telomerase-deficient mutant with deleted *RAD51;* *est2*Δ *G*ALpr-*STN1* - telomerase-deficient mutant with *STN1* expression controlled by GALpr; *rad51*Δ GALpr-*STN1* - mutant with deleted *RAD51* and *STN1* expression controlled by GALpr; *est2*Δ *rad51*Δ GALpr-*STN1* - telomerase-deficient mutant with deleted *RAD51* and *STN1* expression controlled by GALpr.

	YPD		YPGal	
Mutants	Number of DOs	% DOs	Number of DOs	% DOs
*est2*Δ	0/10 DOs	0	0/10 DOs	0
*est2*Δ *rad51*Δ	3/10 DOs	30	1/8 DOs	12.5
*est2*Δ GALpr-*STN1*	6/10 DOs	60	8/10 DOs	80
*rad51*Δ GALpr-*STN1*	0/10 DOs	0	0/10 DOs	0
*est2*Δ *rad51*Δ GALpr-*STN1*	5/20 DOs	25	15/20 DOs	75

Results for AKY3 strain also did not clearly indicate that overexpression of *STN1* was associated with the inability of yeast cells to form type II survivors. The same mutants were streaked as in the case of AKY4, the only difference being that mutants were streaked on YPD+Cu, instead of YPGal plates. Single mutants formed survivors as expected and no DOs were observed. No DOs of *est2*Δ *rad51*Δ mutant were seen on YPD plates, but once again DOs were present on YPD+Cu plates. The difference in the DO presence on YPD and YPD+CU plates was puzzling because this mutant should be able to form type II survivors on both types of plates. In the case of *est2*Δ CUpr-*STN1*, no DOs were present on either of the plates. Although *STN1* should not be expressed in mutants grown on YPD plates, the observed result was as expected since CUpr is known to be leaky. Most likely, CUpr was leaky thus allowing the production of a certain amount of Stn1. In *rad51*Δ CUpr-*STN1* mutant telomerase was active so the percentage of DOs was equal to 0. The observed increase in the percentage of triple mutant DOs correlated with the higher expression of *STN1*. Unfortunately, as was the case with the results for AKY4 strain, other results

(particularly the one for *est2*Δ *rad51*Δ mutant) made it impossible to conclude whether overexpression of *STN1* prohibited type II survivor formation.

Table 12. Single-colony streaking assay performed with mutants of indicated genotype, derived from the sporulation of AKY3. Telomerase-deficient mutants were examined for the ability to bypass senescence by survivor formation. *rad51*Δ GALpr-*STN1* mutant with functional telomerase is used as a control. DOs - dropouts, i.e. mutants that senesced and were not able to form survivors; *est2*Δ - telomerase-deficient mutant; *est2*Δ *rad51*Δ - telomerase-deficient mutant with deleted *RAD51; est2*Δ CUpr-*STN1* - telomerase-deficient mutant with *STN1* expression controlled by CUpr; *rad51*Δ CUpr-*STN1* - mutant with deleted *RAD51* and *STN1* expression controlled by CUpr; *est2*Δ *rad51*Δ CUpr-*STN1* - telomerase-deficient mutant with deleted *RAD51* and *STN1* expression controlled by CUpr.

	YPD		YPD+Copper	
Mutants	Number of DOs	% DOs	Number of DOs	% DOs
*est2*Δ	0/10 DOs	0	0/10 DOs	0
*est2*Δ *rad51*Δ	0/6 DOs	0	5/8 DOs	62.5
*est2*Δ CUpr-*STN1*	0/10 DOs	0	0/10 DOs	0
*rad51*Δ CUpr-*STN1*	0/10 DOs	0	0/10 DOs	0
*est2*Δ *rad51*Δ CUpr-*STN1*	3/8 DOs	37.5	8/13 DOs	61.5

3.5.3 Liquid senescence assay with AKY4 strain

The effect of *STN1* overexpression on type II survivor formation was also examined with liquid senescence assay. As opposed to single-colony streaking assay, senescence assay in liquid cultures enables control over additional conditions that could otherwise influence the final outcome of the assay. After tetrad dissection, the following mutants were identified and used in liquid senescence assay: single (*est2*Δ), double (*est2*Δ *rad51*Δ and *est2*Δ GALpr-*STN1*) and triple (*est2*Δ *rad51*Δ GALpr-*STN1*) mutants. Four cultures of each of the mutants were grown at the beginning of the assay. Along with these mutants, WT and GALpr-*STN1* controls were also included in the study. Two cultures of every control were prepared.

In total, cell density of 18 different cultures was measured each day for 10 days. Results were entered into a graph (Figure 10), with the x-axis corresponding to the number of PDs and the y-axis corresponding to the cell density. Each function represents all of the cultures of a particular mutant or control. The functions of controls did not show any major changes in the cultures' cell density. It was noticed that overexpression of *STN1* had a slightly negative effect on cell growth, because WT grew better and reached higher density values than GALpr-*STN1*. All of the other functions, which described the growth of *est2*Δ, *est2*Δ *rad51*Δ, *est2*Δ GALpr-*STN1* and *est2*Δ *rad51*Δ GALpr-*STN1* mutants, could be divided into three different phases. The first phase represented the decrease in cell density as a consequence of the mutants entering replicative senescence due to telomerase deficiency. After the critical point was reached (the point of the lowest cell density on the graph), survivors formed in all of the cultures and cell density started to rise. The second phase included a progressive rise in cell density, accompanied by survivors overtaking the cultures. The last phase was the plateau phase, in which no or very small changes in cell density were observed. The plateau phase was reached because survivors kept dividing until the cultures no longer contained enough nutrients to sustain the growth of additional survivors. It can be seen from the graph that survivors formed a bit later in the case of *est2*Δ mutant. Also the first phase lasted longer in the case of *est2*Δ than with the other mutants. All of the functions eventually reached a plateau, indicating a similar maximum cell density value for all mutants. Most importantly, the results of this assay suggested that all the mutants were able to form survivors. The results for *est2*Δ, *est2*Δ *rad51*Δ, *est2*Δ GALpr-*STN1*mutants turned out as was expected. The result for *est2*Δ *rad51*Δ GALpr-*STN1* was quite interesting since it suggested that overexpression of *STN1* was not linked to inability of cells to form type II survivors. In order to confirm these results, the type of survivors was validated with Southern blot.

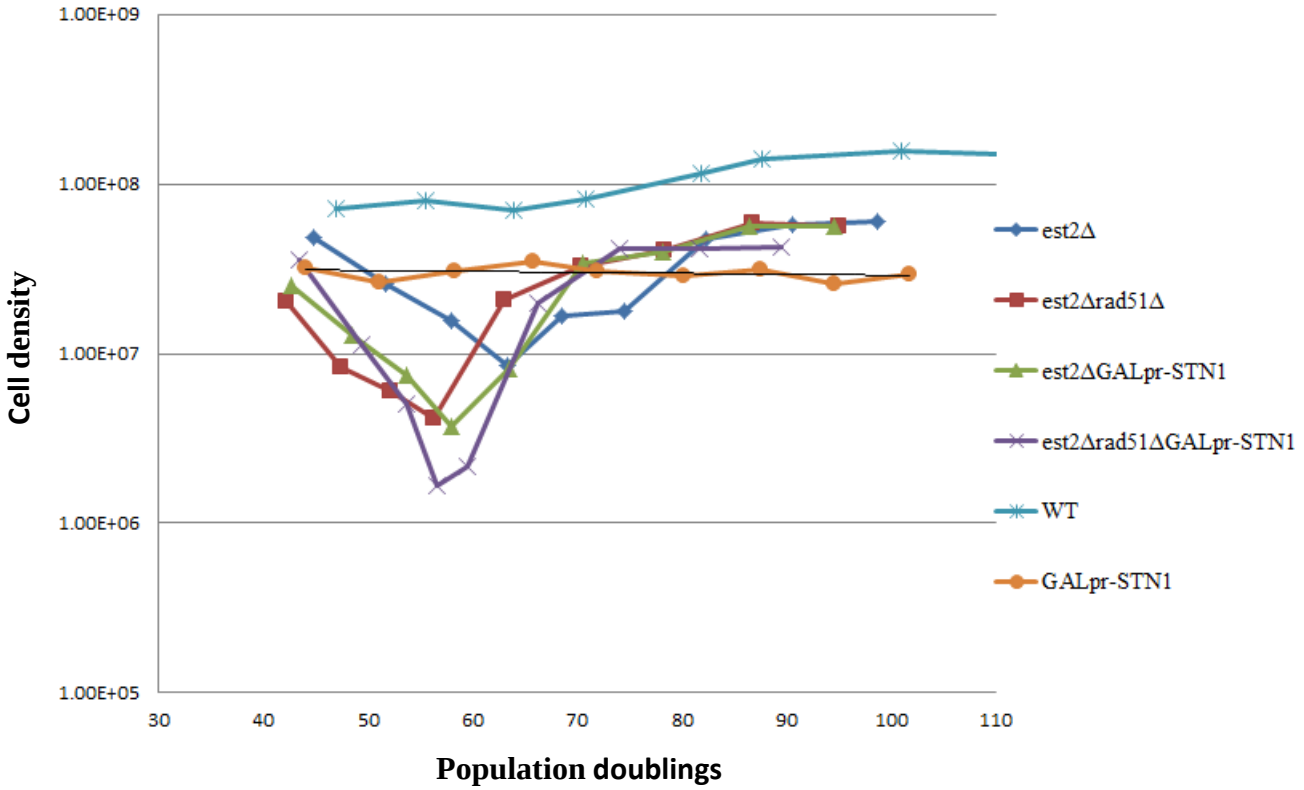

Figure 10. *STN1* overexpression does not prevent type II survivor formation. Growth rates were monitored in liquid culture by serial passaging of haploid meiotic progeny of the indicated genotypes, derived from the sporulation of AKY4. *est2*Δ - telomerase-deficient mutant; *est2*Δ *rad51*Δ - telomerase-deficient mutant with deleted *RAD51*; *est2*Δ GALpr-*STN1* - telomerase-deficient mutant with *STN1* expression controlled by GALpr; *est2*Δ *rad51*Δ GALpr-*STN1* - telomerase-deficient mutant with deleted *RAD51* and *STN1* expression controlled by GALpr; WT - wild type; GALpr-*STN1*- mutant with *STN1* expression controlled by GALpr .

3.5.4 Telomere Southern blot of AKY4 strain mutants

By performing a telomere Southern blot, it is possible to determine whether survivors are type I or II. Samples taken from liquid senescence assay cultures that were collected on day 0 and day 9 were used for Southern blot. These particular samples were used because they represented the starting point and the end point of liquid senescence assay, reflecting the overall changes in cultures accumulated during the assay. It was expected that on day 0 there would be no survivors, while on day 9 survivors would have overtaken the cultures. gDNA was isolated from the day 0 and day 9 samples and digested O/N with *Xho*I. Telomere Southern blot was performed

according to the procedure described in section 2.8. A picture of the developed membrane is given in Figure 11. Samples taken on days 0 and 9 originating from the same mutant culture were positioned adjacent to each other. For the controls, only samples taken from one culture were analyzed. In contrast, samples taken from two different cultures in the case of *est2*Δ and *est2*Δ *rad51*Δ mutants and four different cultures in the case of *est2*Δ GALpr-*STN1* and *est2*Δ *rad51*Δ GALpr-*STN1* mutant were analyzed. The loaded amount of DNA was the same for all of the samples.

In the case of GALpr and WT controls, survivors were not present in the cultures. Bands corresponding to the sample taken on day 0 were slightly weaker than the bands corresponding to the samples from day 9. This was probably due to slower growth of cells, which had to adapt to new conditions in liquid cultures.

Survivor formation was not detected in day 0 samples of *est2*Δ mutant. Results for the day 9 samples of *est2*Δ mutant indicated that type II survivors were present in the cultures even if this mutant should have been able to form both types of survivor. The reason why type II and not type I survivors were detected in the sample had to do with the ability of type II survivors to grow at faster rates than type I.

As was the case with the controls and *est2*Δ mutant, no survivor formation was detected in day 0 samples of *est2*Δ *rad51*Δ mutant. As mentioned before, *est2*Δ *rad51*Δ mutant should be able to form only type II survivors. Indeed, the band pattern suggesting type II survivor formation was observed in both day 9 samples.

All of the samples of *est2Δ GALpr-STN1* mutant were organized in one sequence, with the samples from one and the same culture adjacent to each other. The sample taken on day 0 from the first culture on the left was excluded from the assay because the concentration of DNA was too low for it to be successfully detected. While survivors were not detected in any day 0 sample, type II survivors formed in all of the day 9 samples. This result suggested that mutants in which *STN1* was overexpressed could form type II survivors. *est2*Δ *GALpr-STN1* should have been able to form type I survivors as well. Type I survivors had probably formed in the culture earlier than type II but at some point during the assay type II survivors overtook the culture.

The results for *est2*Δ *rad51*Δ GALpr-*STN1* mutant were similar to the ones obtained for *est2*Δ GALpr-*STN1* mutant. While survivor presence could not be clearly seen in day 0 samples, type II survivors were detected in all of the day 9 samples. This result, together with the one for *est2*Δ GALpr-*STN1* mutant, showed that mutants with *STN1* overexpression were capable of forming type II survivors.

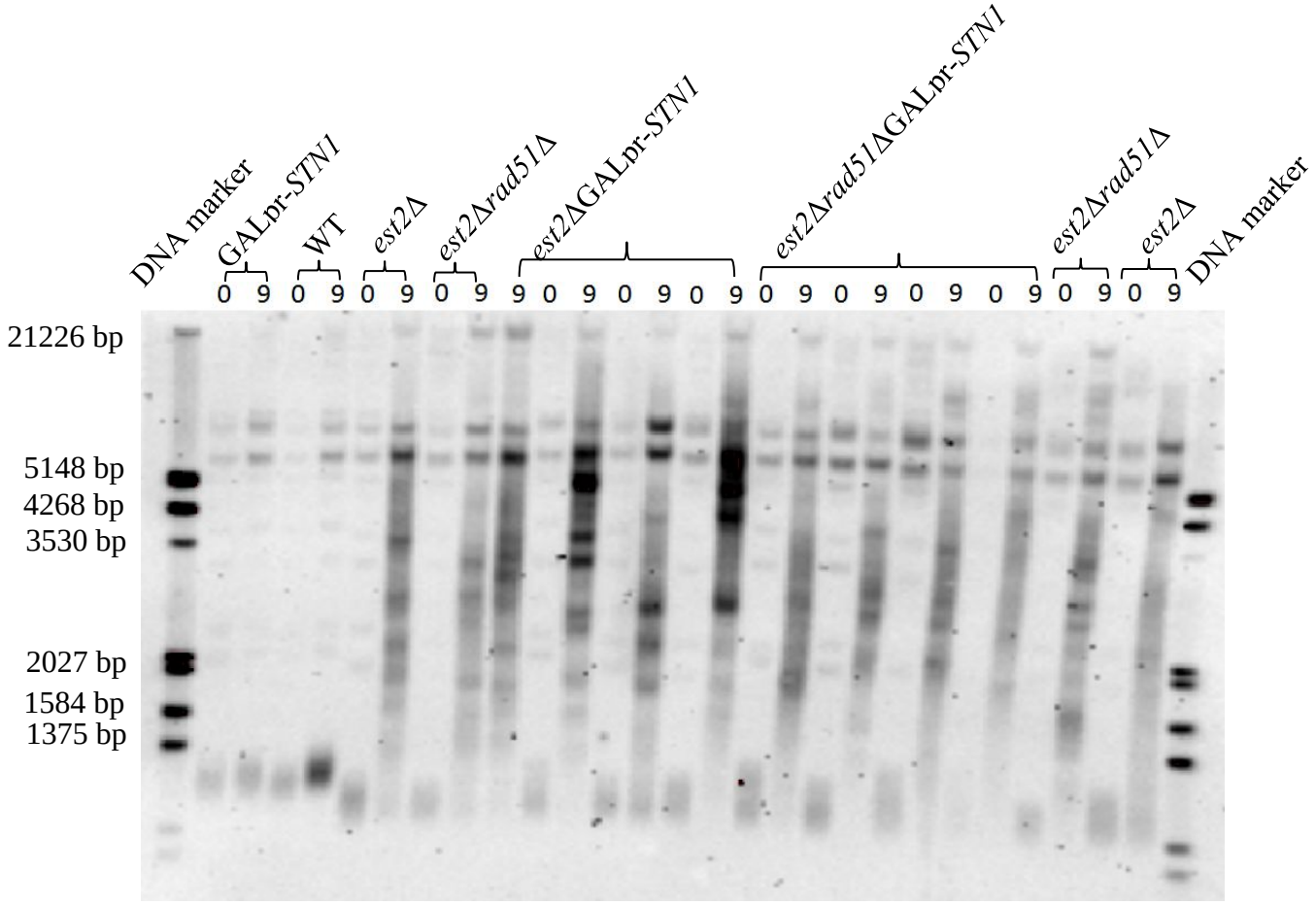

Figure 11. Telomere Southern blot performed with samples from days 0 and 9 of cultures grown in YPGal from liquid senescence assay (cf. section 3.5.3). Type of the formed survivors was determined according to the band pattern. Type I survivors exhibit strong hybridization at 5.2 kb and 6.7 kb, due to the amplification of the subtelomeric Y' elements. Type II survivors exhibit hybridization at many positions due to the diverse lengths of their terminal fragments. These fragments consist of $TG_{1\text{-}3}$ telomeric repeats, which are amplified in type II survivors. GALpr-*STN1*- mutant with *STN1* expression controlled by GALpr; WT - wild type; *est2*Δ - telomerase-deficient mutant; *est2*Δ *rad51*Δ - telomerase-deficient mutant with deleted *RAD51; est2*Δ GALpr-*STN1* - telomerase-deficient mutant with *STN1* expression controlled by GALpr; *est2*Δ *rad51*Δ GALpr-*STN1* - telomerase-deficient mutant with deleted *RAD51* and *STN1* expression controlled by GALpr.

The fact that the mutants in which *STN1* was overexpressed were able to form type II survivors did not necessarily exclude the possibility of high concentrations of Stn1 disrupting the genesis of these survivors. Based on the results presented here it was clear that *STN1* overexpression could not completely block type II survivor formation but it could have still interfered with the events leading to generation of this type of survivor. In other words, *STN1* overexpression might have blocked type II survivor formation to a certain degree, allowing sporadic successful formation. In that respect, it is possible that the detected type II survivors (in the case of *est2*Δ GALpr-*STN1* and of *est2*Δ *rad51*Δ GALpr-*STN1* mutants) all originated from a small number of initially formed type II survivor cells. Since the samples in which type II survivors were detected were gathered 9 days after the beginning of the assay, even a small number of type II survivors would have had enough time to overtake the cultures.

In order to confirm this hypothesis, another telomere Southern blot was performed (Figure 12). This time, day 9 samples were replaced with samples in which survivor formation was first detected. These samples were taken at the point in time when cell density started increasing due to survivor formation. Survivors present in these samples were referred to as “early survivors”. Day 0 samples were excluded from this analysis. In the case of controls, the same principle could not be followed since survivor formation did not take place. Day 5 samples were then selected for analysis since most samples from other mutants were taken on the fifth day. Samples of controls and mutants were arranged on the membrane in the same order as on the previous one.

In the case of the controls, several additional bands could be observed along with the ones corresponding to the length of 5.5 kb or 6.5 kb. These bands represented the telomeres which did not contain Y’ subtelomeric elements.

Two *est2*Δ mutant samples were analyzed and it was determined that type II survivors were predominant in one sample, while type I were in the other. This result suggested that type I survivors were indeed formed in the culture, which was soon after overtaken with type II survivors. Furthermore, type II survivors were detected in all of the other samples which suggested that type II survivors were formed early in the culture. This type of survivor was present in *est2*Δ *GALpr-STN1* and *est2*Δ *rad51*Δ *GALpr-STN1* cultures soon after the increase in cell density was observed. Thus, it was unlikely that the type II survivors detected at day 9 originated from a small number of initially formed survivors. These results showed that type II

survivor formation was not severely affected by *STN1* overexpression. Nevertheless, the possibility of excess Stn1 partially disturbing the events leading to genesis of this type of survivor could not be completely rejected.

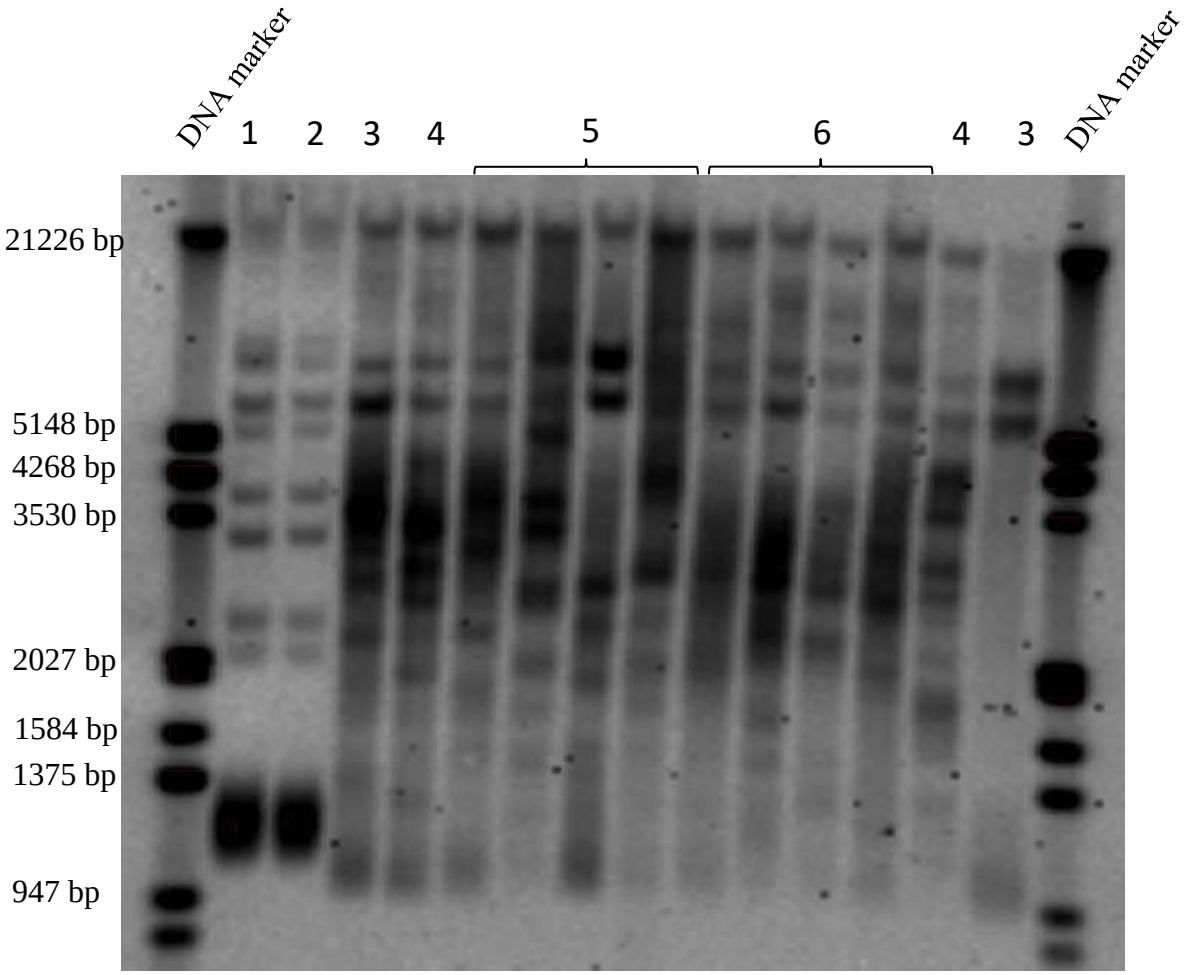

Figure 12. Telomere Southern blot performed with samples of cultures grown in YPGal from liquid senescence assay (cf. section 3.5.3). A majority of samples were gathered on day 5 or day 6, representing the time point at which early survivors were formed. Type I survivors exhibit strong hybridization at 5.2 kb and 6.7 kb, due to the amplification of the subtelomeric Y' elements. Type II survivors exhibit hybridization at many positions due to the diverse lengths of their terminal fragments. These fragments consist of TG_{1-3} telomeric repeats, which are amplified in type II survivors. 1 - GALpr-*STN1*; 2 - WT; 3 - *est2*Δ; 4 - *est2*Δ *rad51*Δ; 5 - *est2*Δ GALpr-*STN1*; 6 - *est2*Δ *rad51*Δ GALpr-*STN1*.

3.5.5 Effect of *STN1* overexpression on telomere length

It has been reported in the literature that telomeres are maintained at shorter length in mutants with *STN1* overexpression. Dahlseid and coworkers (2002) showed that *STN1* overexpression reduced telomere length to the same extent as was observed for NMD mutants.

This assay investigated whether *STN1* overexpression with GALpr would have an impact on telomere length. In order to do so, telomere length of WT and GALpr-*STN1* mutant was analyzed with telomere Southern blot and the respective results were compared to each other. Prior to Southern blot analysis, tetrad dissection was performed and haploid WT and GALpr-*STN1* colonies were identified. WT and GALpr-*STN1* cultures were grown O/N in YPD and YPGal at 30^{o}C. The next day gDNA was isolated and digested O/N with *Xho*I. Telomere Southern blot was performed according to the protocol described in section 2.8. Results are shown in Figure 13. It can be seen in the picture that the band pattern and the signal strength were similar for all of the samples. In addition, bands representing telomere terminal fragments (1-1.3 kb) were very much alike, suggesting that *STN1* overexpression is not associated with shorter telomere phenotype. Such a result does not correspond to those published in the literature or to the proposed function of Stn1 in inhibiting telomerase recruitment at telomeres. A possible explanation of the observed result is that the performed assay was not sensitive enough to detect small changes in telomere length between WT and GALpr-*STN1* mutant. Another explanation could be that *STN1* was not as strongly overexpressed as in the other studies. If that was the case, maybe the change in telomere length could not be detected or not enough time passed for additional Stn1 to have an impact on it.

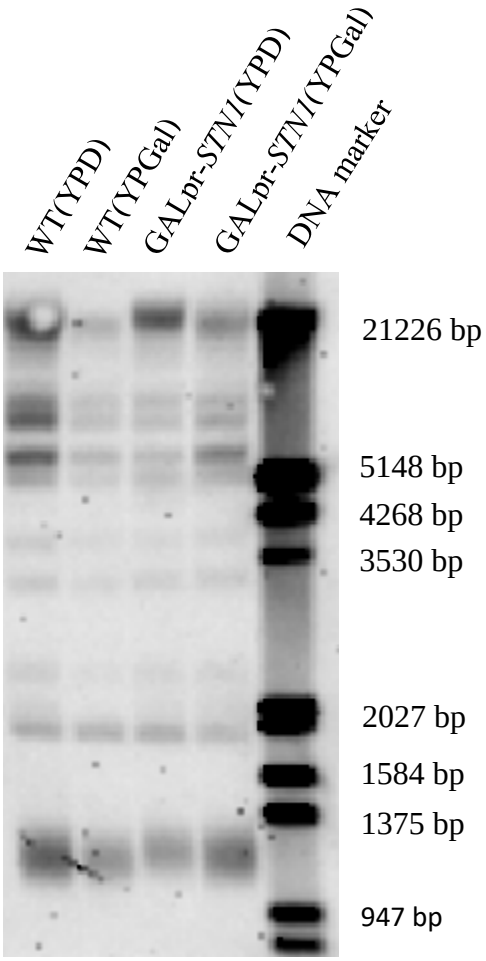

Figure 13. Telomere Southern blot of WT and *GAL*pr-*STN1* mutant grown in YPD and YPGal. The bands between 1-1.3 kb represent terminal telomere fragments. These fragments contain TG_{1-3} repeats that are elongated by the action of telomerase. In the case of both WT and GALpr-*STN1* mutants terminal telomere fragments are of similar size, suggesting that telomeres are not maintained at shorter length when *STN1* is overexpressed. WT - wild type; GALpr-*STN1*- mutant with *STN1* expression controlled by GALpr.

3.6. Impact of *RAD51* overexpression on survivor formation

As mentioned in section 1.1, NMD is not strictly confined to the mRNA bearing a premature termination codon. Aside from controlling the levels of telomere related proteins, NMD plays a direct role in regulation of HR. Janke and associates (2016) reported that mRNA levels of *RAD51*, *RAD54*, *RAD55* and *RAD57* are regulated by NMD. As previously described in section 1.3., *RAD51* is involved in type I survivor formation while it is not required for the generation of type II survivors. Overexpression of *RAD51* may thus result in more frequent type I survivor

formation. If Rad51 is present in relatively high quantities, the balance between occurrences of type I and type II survivors could shift toward type I. As a result, the generation of type II survivors could prove rare or even not possible. In order to test this hypothesis, AKY5 and AKY6 strains were constructed. Tetrad dissection enabled the identification of mutants that would later be used for single-colony streaking assay. In the assay, mutants of interest were streaked both on YPD and YPGal plates. Results of single-colony streaking assay for AKY5 strain are shown in Table 13 and for AKY6 strain in Table 14.

As can be seen from the tables, all of the mutants were successful in survivor formation. This indicates that *RAD51* overexpression did not interfere with events leading toward survivor formation. The percentage of DOs was higher than 0 only in the case of *est2*Δ GALpr-*RAD51* mutant grown on YPD plates. A possible explanation for the observed number of DOs is that colonies that did not contain survivors were picked and streaked.

Table 13. Single-colony streaking assay performed with mutants of the indicated genotype, derived from the sporulation of AKY5. Telomerase-deficient mutants were examined for the ability to bypass senescence by forming survivors. GALpr-*RAD51* mutant with functional telomerase is used as a control. DOs - dropouts, i.e. mutants that senesced and were not able to form survivors; *est2*Δ - telomerase-deficient mutant; GALpr-*RAD51* - mutant with *RAD51* expression controlled by GALpr; *est2*Δ GALpr-*RAD51* - telomerase-deficient mutant with *RAD51* expression controlled by GALpr.

	YPD		YPGal	
Mutants	Number of DOs	% DOs	Number of DOs	% DOs
*est2*Δ	0/10 DOs	0	0/9 DOs	0
GALpr-*RAD51*	0/10 DOs	0	0/10 DOs	0
*est2*Δ GALpr-*RAD51*	2/10 DOs	20	0/10 DOs	0

Table 14. Single-colony streaking assay performed with mutants of the indicated genotype, derived from the sporulation of AKY6. Telomerase-deficient mutants were examined for the ability to bypass senescence by forming survivors. GALpr-*RAD51* mutant with functional telomerase is used as a control. DOs - dropouts, i.e. mutants that senesced and were not able to form survivors; *tlc1*Δ - telomerase-deficient mutant; GALpr-*RAD51* - mutant with *RAD51* expression controlled by GALpr; *tlc1*Δ GALpr-*RAD51* - telomerase-deficient mutant with *RAD51* expression controlled by GALpr.

	YPD		YPGal	
Mutants	Number of DOs	% DOs	Number of DOs	% DOs
*tlc1*Δ	0/5 DOs	0	0/5 DOs	0
GALpr-*RAD51*	0/5 DOs	0	0/5 DOs	0
*tlc1*Δ GALpr-*RAD51*	0/10 DOs	0	0/10 DOs	0

Survivor colonies of *est2*Δ GALpr-*RAD51* mutant that formed in the single-colony streaking assay just described were further analyzed. Seven early survivor colonies (formed immediately after the cells started senescing) were taken from both YPD and YPGal plates and grown for 16 hours in 2.5 ml of YPD and YPGal medium respectively. gDNA was then isolated from samples of all the cultures and digested O/N with *Xho*I. Digested gDNA was further analyzed with telomere Southern blot, results of which are shown in Figure 14. gDNA of *est2*Δ GALpr-*RAD51* mutants that had been grown on YPD plates were loaded into lanes 2-8 and that of *est2*Δ GALpr-*RAD51*mutants that had been grown on YPGal plates into lanes 9-15. Results indicated that mutants without *RAD51* overexpression (i.e. the ones grown on YPD) were able to form type II survivors, while mutants in which *RAD51* was overexpressed (i.e. the ones grown on YPGal) formed type I survivors. This result was expected: in mutants grown on YPD plates transcription of *RAD51* was shut down so survivors generated were type II. In *est2*Δ GALpr-*RAD51* mutant grown on YPGal, *RAD51* was expressed and type I survivor formation was thus allowed. Type II survivors were not detected in the mutant grown on YPGal most likely because 16 hour incubation in liquid media did not allow enough time for type II survivors to suddenly form and to take over the culture. It is also possible that the conditions of higher Rad51 concentrations favored type I survivor formation, while not being involved in blocking the formation of type II survivors.

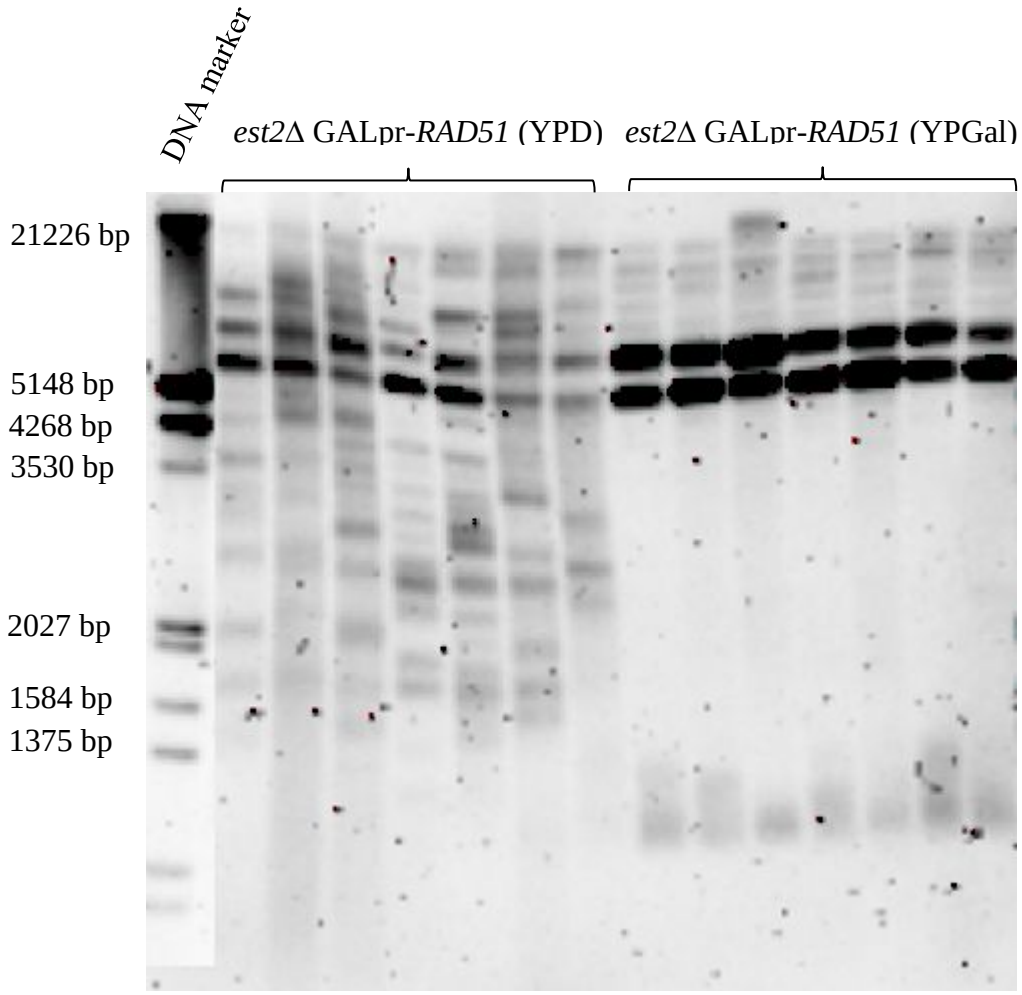

Figure 14. Telomere Southern blot of *est2*ΔGALpr-*RAD51* survivors grown in YPD or YPGal. Type I survivors exhibit strong hybridization at 5.2 kb and 6.7 kb, due to the amplification of the subtelomeric Y' elements. Type II survivors exhibit hybridization at many positions due to the diverse lengths of their terminal fragments. These fragments consist of the $TG_{1\text{-}3}$ telomeric repeats, which are amplified in type II survivors. *est2*Δ GALpr-*RAD51* - telomerase-deficient mutant with *RAD51* expression controlled by GALpr.

4. Discussion

The connection between NMD and survivor formation in *S. cerevisiae* was first established when Hu and coworkers (2013) showed that *UPF1, UPF2, UPF3* and *EBS1* are required for type II survivor formation. The study presented here tested whether mutants in which either *UPF1, UPF2, UPF3* or *EBS1* was deleted would be able to generate type II survivors and then evaluated exactly how NMD would influence type II survivor formation.

Single-colony streaking assays with AKY1, AKY2 and AKY8 strains were performed. With all the three strains, the percentage of DOs was highest for triple mutants, suggesting that *UPF1, UPF3* and *EBS1* were required for type II survivor formation. On the other hand, 20% of *est2*Δ *rad51*Δ *upf1*Δ, 15% of *est2*Δ *rad51*Δ *upf3*Δ and 25% of *est2*Δ *rad51*Δ *ebs1*Δ mutant were able to form survivors. Since *RAD51* was deleted in these mutants, it is highly probable that the formed survivors were type II, but telomere Southern blot would have to be performed in future studies to prove this. If the assumption regarding the survivor type is correct, it is logical to assume that deletion of *UPF1, UPF3* and *EBS1* does not completely block the formation of this type of survivors. The results for *UPF1* and *UPF3* could therefore imply that *UPF2* does not have a specific function in type II survivor formation and that there is a connection between this process and NMD. However, an earlier study in Dr. Chang's laboratory (unpublished data) and a study by Hu and coworkers (2013) suggested that *upf2*Δ mutants were completely incapable of type II survivor formation. At present, I cannot explain this discrepancy and further studies are needed to address this issue. Another point that should be addressed is to what extent the deletion of *UPF1, UPF3* and *EBS1* affects the generation of type II survivors. Observations from single-colony streaking assays made in this study should be confirmed and further explored with liquid senescence assays followed by telomere Southern blots. These assays could provide additional information about the efficiency and frequency of type II survivor formation. Also, by analyzing samples gathered on different days during liquid senescence assay, the time point at which type II survivors form could be determined. By doing so, it could be established whether deletion of *UPF1, UPF3* and *EBS1* disturbs the generation of type II survivors and to what extent.

The results from single-colony streaking assay applying to *est2*Δ, *est2*Δ *upf1*Δ, *est2*Δ *upf2*Δ and *est2*Δ *ebs1*Δ were as expected, with no DOs or only one DO in the case of *est2*Δ *ebs1*Δ mutant.

Surprisingly, the same was not true of the *est2*Δ *rad51*Δ mutant. Although this mutant should have been able to form type II survivors, the percentage of DOs was 60 % (for AKY1) and 40 % (for AKY2 and AKY8). If one takes into account that type II survivors occur less frequently than type I on solid-medium-grown cultures, it is justified to assume that type II survivor colonies may have not been picked and restreaked during single-colony streaking assay. As a result, it seemed as if many est2Δ *rad51*Δ mutants were not able to overcome senescence. This assumption is substantiated by the fact that a considerable number of *est2*Δ *rad51*Δ DOs were observed in the majority of single-colony streaking assays performed in this study and earlier in Dr. Chang's laboratory (unpublished data). Nevertheless, it remains doubtful whether this explanation can account for the observed percentages of *est2*Δ *rad51*Δ DOs. It is thus also possible that the increased number of DOs was linked to deletion of *RAD51* in *est2*Δ background, although the existence of this link is uncertain. All in all, although the percentage of *est2*Δ *rad51*Δ DOs was noticeably high it was still lower than the percentage of triple mutant DOs and thus did not exclude the possibility of *UPF1*, *UPF3* and *EBS1* being involved in the genesis of type II survivors. In order to verify the published data reporting the importance of *UPF2* for type II survivor formation, liquid senescence assay was performed with AKY7. Unexpectedly, survivor formation was detected in all of the cultures of *est2*Δ *rad51*Δ *upf2*Δ mutant. The type of the formed survivors was not checked with telomere Southern blot, but since these mutants could not form type I survivors, a reasonable assumption was that the formed survivors were of type II. Such an outcome counters not only the results reported by Hu and coworkers (2013), but also the one obtained in the laboratory of Dr. Chang. Hu and associates grew 280 telomerase-null mutants with different single gene deletions serially passaged in a liquid medium in order to screen for genes that might affect type II survivor formation. The way in which they performed the assay did not significantly differ from the liquid senescence assay described in this study. The main difference was that Hu and coworkers worked with BY4741 yeast strain background, while the experiments in this study were carried out in W303 strain background. It is not known whether type II survivor formation is differently affected when working in one or the other strain background. However, even if yeast strain background somehow affected the process in the study by Hu et al. (2013), that explanation says nothing about the lack of correspondence between the observations made earlier in the Dr. Chang's laboratory and the ones described in this study. The former were based on liquid senescence assay with YPM10 (*MAT**a**/α est2::URA3/EST2*

rad51::natMX4/RAD51 upf2::kanMX4/UPF2). This strain harbored the same gene deletions as AKY7. Nevertheless, survivor formation was not detected in the cultures of the *est2*Δ *rad51*Δ *upf2*Δ mutant derived from YPM10. Liquid senescence assay in the study reported here was performed under same conditions as the earlier one, the only difference being different origin of the examined mutants. Both YPM10 and AKY7 were obtained by transforming YPM9, and the construction of these strains was checked with PCR. With regard to that, it is puzzling why triple mutants from AKY7 were able to form survivors, when the ones from YPM10 were not. To elucidate this matter, a new AKY7 strain should be constructed and the liquid senescence assay should be repeated with the new strain.

As described in 1.4, we hypothesized that NMD influences type II survivor formation by regulating the level of protein(s) important for telomere biology. As it was pointed out, several telomere related proteins are regulated by NMD, among them Stn1 and Ten1 (Holstein et al., 2014). We decided to focus our attention on *STN1* since observations reported earlier (cf. section 1.5.) made it reasonable to assume that *STN1* could be the link between NMD and type II survivor formation. Two strains were therefore constructed, AKY3 and AKY4, in which expression of *STN1* was put under the control of CUpr and GALpr respectively. Single-colony streaking assay was performed to assess whether *STN1* overexpression would interfere with type II survivor formation. Unfortunately, neither the results for AKY3 nor those for AKY4 provided a clear indication of a connection between *STN1* overexpression and the inability of cells to form type II survivors. In the case of AKY4, overexpression of *STN1* resulted in an increase in the number of *est2*Δ *rad51*Δ *GALpr-STN1* DOs. The DO percentage for the mutant grown on YPD was 25% and it was 75% for the mutant grown on YPGal. This observation could be interpreted as if the excess Stn1 impaired type II survivor formation but did not completely block it, since 25% of triple mutants grown on YPGal were able to bypass senescence by forming survivors. Nevertheless, other results, especially the one for *est2*Δ GALpr-*STN1* mutant, do not support this claim. Surprisingly, the percentage of *est2*Δ GALpr-*STN1* DOs (80%) was higher than the one for triple mutants (75%). Since *est2*Δ GALpr-*STN1* mutant should have been able to form type I survivors, which was not the case with triple mutant, one would have expected that more DOs would be observed in the case of triple mutant. Still, it is unknown whether overexpression of *STN1* affects type II survivor formation in *est2*Δ in a different way than in *est2*Δ *rad51*Δ

background. In addition, the literature does not rule out the possibility of *STN1* overexpression interfering with type I survivor formation. The high percentage of *est2*Δ GALpr-*STN1* DOs could thus be explained by *STN1* overexpression affecting type I survivor formation and brings about a stricter prohibition of type II survivor generation than in the *est2*Δ *rad51*Δ background. On the other hand, the results for the *est2*Δ CUpr-*STN1* do not support this view since no DOs of this mutant were present on YPD+Cu plates, although this difference may be due to different levels of overexpression using the two promoters.

It could be assumed that the increase in the number of *est2*Δ *rad51*Δ CUpr-*STN1* DOs was a consequence of *STN1* overexpression but the result for *est2*Δ *rad51*Δ mutant in particular does not support such a claim. Since the percentage of DOs was almost the same for *est2*Δ *rad51*Δ and *est2*Δ *rad51*Δ CUpr-*STN1* mutant, it is not clear whether overexpression of *STN1* disturbed type II survivor formation. It is puzzling why the number of *est2*Δ *rad51*Δ DOs was so high since this mutant should have been able to form type II survivors. It is also unclear why no *est2*Δ *rad51*Δ DOs were observed on YPD plates, when their composition differs from YPD+Cu plates only in that they do not contain copper. As discussed above, one explanation could be that survivor colonies were not picked and restreaked during single-colony streaking assay because of the low incidence of type II survivor formation on solid-medium-grown cultures.

With *STN1* being an essential gene, *est2*Δ GALpr-*STN1*, *rad51*Δ GALpr-*STN1* and *est2*Δ *rad51*Δ GALpr-*STN1* mutants should not have been able to grow on YPD plates since *STN1* was not expressed. Most likely, GALpr was leaky thus allowing the production of sufficient Stn1 to enable the growth of these mutants. The results of the experiment described in section 3.5.1 support this proposition. As can be seen in Figure 9, GALpr-*STN1* mutant was able to grow on YPD plate, although less efficiently than on YPGal plate. In the case of AKY3, *est2*Δ GALpr-*STN1*, *rad51*Δ GALpr-*STN1* and *est2*Δ *rad51*Δ GALpr-*STN1* mutants were also able to grow on YPD. Such an observation was expected, since it is known that CUpr is even leakier than GALpr. The reason why results of single-colony streaking assays with AKY3 and AKY4 are inconclusive may be connected with the way in which such assays are carried out. Although one colony is streaked each time, it is very unlikely that all the colonies picked within one assay are of the same size. In other words, the amounts of cells transferred to a new section of plates differ. Furthermore, the streaked colonies may consist of a varying number of senescing or survivor cells or even cells in a different phase of the cell cycle. In addition, telomeres of the cells making

up various colonies are not necessarily of the same length. In case that a colony containing cells with insufficiently long telomeres is selected, this may result in no colony formation on the new section. On the other hand, if a colony with cells having longer telomeres is selected, new colonies can form and additional time for survivor cells to emerge is provided. If in the next streaking cycle the newly formed survivor colony is picked, this could greatly influence the final result of the assay. With this in mind, it is clear that certain conditions cannot be controlled when single-colony streaking assay is performed. It is thus possible that the results of the assays in my study were influenced by the way they were carried out.

In order to further evaluate the idea that excess Stn1 prevents type II survivor generation, liquid senescence assay was performed with AKY4. Results showed that *est2*Δ, *est2*Δ *rad51*Δ, *est2*Δ GALpr-*STN1* and *est2*Δ *rad51*Δ GALpr-*STN1*mutants were able to form survivors. All of the mutants first started senescing until the critical point was reached and then the cultures were "repopulated" with survivor cells. The fact that survivor formation was detected in *est2*Δ *rad51*Δ GALpr-*STN1* mutant cultures suggested that *STN1* overexpression does not prevent type II survivor formation – the survivors had to be of that type, since this mutant is believed not to be able to form type I survivors. This was confirmed with telomere Southern blot. First, Southern blot with samples from day 0 and day 9 was performed. The samples were chosen because they reflect the overall change that took place during liquid senescence assay. In addition, between the first and the last day enough time was provided for type II survivors to form. As expected, survivor formation was detected only in day 9 samples. Survivors in the *est2*Δ and *est2*Δ *rad51*Δ samples were type II. With the former, type I survivor formation most likely preceded type II survivor formation, which overtook the cultures afterwards. The most significant results were the ones for *est2*Δ GALpr-*STN1* and *est2*Δ *rad51*Δ GALpr-*STN1* mutants. Type II survivors were detected in all of the day 9 samples of these mutants indicating that the *STN1* overexpression did not prevent type II survivor formation. Nevertheless, this observation did not exclude the possibility that overexpression of *STN1* interfered with type II survivor generation. In other words, high levels of Stn1 may only partially block formation of these survivors thus limiting the likelihood of their generation. As mentioned before, it is thus possible that the detected type II survivors (in the case of *est2*Δ GALpr-*STN1* and of *est2*Δ *rad51*Δ GALpr-*STN1* mutants) all originated from a small number of initially formed type II survivor cells. In order to test this,

telomere Southern blot with samples of early survivors was performed. Type II survivor formation was detected in both the *est2*Δ GALpr-*STN1* and *est2*Δ *rad51*Δ GALpr-*STN1* samples, suggesting that these survivors were already present in the cultures soon after the observed increase in cell density. Since type II survivors were detected in the cultures at this early stage, the explanation that the type II survivors detected at day 9 originated from only a small number of initially formed survivors does not seem well-founded. It seems thus that *STN1* overexpression did not significantly disturb type II survivor formation. Still, the hypothesis that *STN1* overexpression blocks the generation of this survivor type to a certain degree was thereby not completely disproven. Unfortunately, the limitations of liquid senescence assay render it impossible to provide an unequivocal answer to this issue. Even if with this kind of assay more conditions can be controlled than with single-colony streaking assay, it still has its limitations. It is thus not sensitive enough to exclude the possibility of excess Stn1 partially disturbing generation of type II survivors. As described earlier in this section, we tried to prove this proposition by specifically analyzing the samples taken at the time point closest to the earliest detected survivor formation. Even though these were the first samples gathered after a noticeable rise in cell density, they did not necessarily reflect the situation in cultures right after the formation of earliest survivors. In other words, the assay in general does not provide the option to gather samples immediately after the generation of survivors. Thus, even the early samples that were analyzed may not have been adequate to enable us to notice the effects of *STN1* overexpression on the formation of type II survivors. This claim is corroborated by the fact that only type II survivors were detected in the early samples of *est2*Δ GALpr-*STN1* mutant. Since this mutant should have been able to form type I survivors (which generally arise more frequently in cultures than type II), it was expected that these survivors would be the first to form. The fact that type I survivors were not detected in any of the *est2*Δ GALpr-*STN1* early samples, suggests that enough time had passed for type II survivors to form and overtake the cultures. This circumstance would thus support the claim that the samples did not correspond to the time point right after the initial survivor formation.

Another flaw of liquid senescence assay is the inability to secure consistent serial passaging of the content containing haploid meiotic progeny. In other words, it is not possible to ensure that the transferred samples will contain a corresponding number of viable, senescing and survivor cells, which can affect the outcome of the assay. That might also be an explanation why the

results of liquid senescence assay were different when performed with YPM10 and AKY4 strains.

In addition to analyzing the effects of *STN1* overexpression on type II survivor formation it was worth checking whether overexpression of *STN1* with GALpr would bring about a decrease in the length at which telomeres were maintained. In order to do so, telomeres of WT and GALpr-*STN1* mutant grown O/N in YPD and YPGal were analyzed. Surprisingly, the obtained result did not align with the observations described by Dahlseid and coworkers (2002) about *STN1* overexpression reducing telomere length to the same extent as was observed for NMD mutants. The length of telomere terminal fragments was much the same in the case of both the WT and GALpr-*STN1* mutant grown in YPD and YPGal. Such a result suggests that overexpression of *STN1* does not cause short telomere phenotype, which would also contradict the proposed role of Stn1 as a negative regulator of telomerase (Grandin et al., 2000). The fact that the expected phenotype was not observed can be explained by one of the following circumstances. First, the performed telomere Southern blot analysis might not have been sensitive enough to distinguish small differences in the length of telomere fragments. Dahlseid et al. (2002) describe that the length of the 0.8 kb long terminal telomere fragment was ~43+ /- 19 bp shorter in mutants with *STN1* overexpression compared to WT. The Southern blot performed in this study may have failed to spot such small changes even if they were actually present. Such changes in telomere length could be detected by telomere PCR. Secondly, the concentration of cellular Stn1 in GALpr-*STN1* mutant might not have been high enough to cause the expected phenotype. Since GALpr-*STN1* construct is uncharacterized, the exact degree of *STN1* overexpression remains to be determined. In their work, Dahlseid et al. (2002) used yeast 2 µm vectors containing multiple copies of *STN1* with its native promoter. It is possible that the levels of mRNA, as well as Stn1, were higher in cells transformed with 2 µm vectors compared to the ones with an integrated GALpr-*STN1* construct. In other words, overexpression of *STN1* with GALpr might not be strong enough to cause the maintenance of telomeres at a shorter length than in WT. If the levels of *STN1* expressions indeed were different in the two studies, more time could have been required for Stn1 to confer its effect on telomere phenotype in AKY4. The difference in the length of telomere terminal fragments might thus have been more perceptible if the incubation time of WT and GALpr-*STN1* mutant cultures had been increased.

In future work it should be determined how much the steady-state of Stn1 increases in mutants containing GALpr-*STN1* construct. It would also be necessary to find out whether Stn1 level increase in these mutants generally equals or exceeds the magnitude of the Stn1 increase in *upf*Δ mutants. This can be done by performing northern blot analysis and establishing whether the levels of *STN1* mRNA are increased in GALpr-*STN1* mutants. Another approach may be to measure and compare the change in Stn1 concentration in GALpr-*STN1* and in *upf*Δ mutants. This can be achieved by creating a construct in which *STN1* is fused with a certain tag sequence. The difference in Stn1 levels in mutants of interest could be determined by Western blot analysis.

Aside from controlling the levels of telomere related proteins, NMD plays a direct role in regulation of HR. It is therefore possible that NMD regulates type II survivor formation not through regulation of a certain telomere related protein but by affecting the levels of a specific protein involved in HR events that lead to survivor formation. One of the HR genes controlled by NMD is *RAD51*, whose role as a potential link between NMD and type II survivor formation was explored in this study. *RAD51* is required for generation of type I survivors since it is involved in amplification of Y' subtelomeric elements (Rubelj I., 2014-2015). Janke et al. (2016) have reported that mRNA levels of *RAD51* are increased in mutants with non-functional NMD pathway. We hypothesized that *RAD51* overexpression might promote type I survivor formation and that type II survivor generation would consequently be suppressed. In order to test that, several assays were performed with AKY5 and AKY6 strains. The single-colony streaking assay indicated that mutants with overexpressed *RAD51* were able to form survivors. As described in section 3.6., the type of the survivors was established with telomere Southern blot. As expected, mutants without *RAD51* overexpression (i.e. the ones grown on YPD) were able to form type II survivors, while mutants in which *RAD51* was overexpressed (i.e. the ones grown on YPGal) formed type I survivors. Although only type I survivors were detected in all seven samples of the mutant grown on YPGal, it is possible that not enough time was provided for type II survivors to emerge and overtake the cultures. Furthermore, it is possible that the high concentration of Rad51 blocked or delayed type II survivor formation. In future work, liquid senescence assay should be performed with AKY5 or AKY6 strain to clarify this issue.

5. Conclusion

The study described here was done within the framework of a larger project carried out in Dr. Chang's laboratory at ERIBA, which focused on the identification and characterization of genes important for type II survivor formation in *S. cerevisiae*. A genome-wide screen was performed at an early stage of the project and one of the identified hits was *UPF2*, a gene involved in NMD. My study focused on what was a logical next step – examining the dependence of type II survivor formation on NMD in more detail. Although only *UPF2* was identified as a hit in the genome-wide screen, the study seems to suggest that type II survivor formation is in some extent also dependent on *UPF1* and *UPF3*. This could therefore imply that *UPF2* does not have a specific function in type II survivor formation and that there is a connection between this process and NMD. This conclusion is also supported by the results reported by Hu et al. (2013). Surprisingly, results obtained in this study do not indicate that *UPF2* is absolutely required for type II survivor formation. Nevertheless, as described in more detail in chapter 4, it is possible that *UPF2* partially blocks this process. Another finding of this study is that *EBS1*, a global inhibitor of translation, seems to be required for type II survivor formation, even if it is not one of the three genes involved in NMD. The study also pursued the idea that *STN1* might be a potential link between NMD and type II survivor formation. The assays performed to examine this proposition have not confirmed it since their results are either inconclusive or indicating that *STN1* is not a link. Nevertheless, it remains to be determined whether overexpression of *STN1* can partially impair type II survivor formation. In the last stage of the study, stemming from the claim in literature that NMD regulates mRNA levels of genes involved in HR, we tested whether *RAD51* could represent the connection between NMD and inability of cells to form type II survivors. Although type II survivors were not detected in mutants with *RAD51* overexpression, the assumption has to be further examined before a final conclusion can be reached. By way of a general conclusion we can say that, although the observations made in this study suggest that NMD is involved in the regulation of type II survivors, the exact link remains to be discovered.

6. Literature

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Curriculum vitae

PERSONAL INFORMATION

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EDUCATION

since September 2014	**Graduate student (MS level) of Molecular Biology** All the courses of the two-year Master's program completed, currently awaiting defense of the master's thesis
2011 – 2014	**Undergraduate studies of Molecular biology**, Faculty of Science, University of Zagreb (degree obtained: univ. bacc. biol. mol.)
September 2007 – June 2011	Student at Classical Grammar School PKG, Zagreb

EXTRA CURRICULAR PROFESSIONAL TRAINING

March – October 2016

- Working at the **European Research Institute for the Biology of Ageing** in Groningen in the group of assoc. prof. **Michael Chang**, completing the Master thesis project **"Investigating the link between nonsense-mediated mRNA decay and survivor formation in *S. cerevisiae*"**

July – August 2015

- Participating in **The International Summer School (ISS)** focusing on **"Gene Regulation, Epigenetics and Genome Stability"**. Working in Dr. **Petra Beli's lab** at the Institute of Molecular Biology in Mainz on the project **"Exploring the interaction between FEN1 and Cullin-RING ligases"**

July 2014

- Working in prof. **Klaus Förstemann's laboratory** for one month at the Gene Center of the Ludwig-Maximilian University in Munich; learning about prof. Förstemann's work (Biogenesis and function of small RNAs) and doing practical work (form of a mini project)

March – May 2014

- Doing laboratory skill training and assisting in the lab of assoc. prof. **Ivana Ivančić Baće** at the University of Zagreb, Department of Molecular biology (the project involved studying CRISPR/Cas mechanism in *E. Coli*)

July 2013

- 7-day visit to prof. **Xavier Perret's** laboratory at the University of Geneva, Department of Botany and Plant biology; observing and learning about prof. Perret's work (Genetics of symbioses between *Sinorhizobium fredii* NGR234 and legumes), spending time with his team and doing some basic practical work under their supervision

July 2012

- 5-day visit to prof. **David Shore's** laboratory at the University of Geneva, Department of Molecular Biology; observing and learning about prof. Shore's work (establishing relationship between chromosome structure and the process of gene regulation, DNA replication, chromosome segregation), spending time with his team and doing some basic practical work in lab under their supervision

July 2010

- **Summer School of Science S3++**, Višnjan, Croatia (team project in biology „Finding Antibiotic Resistance Genes Using Phyletic Profiling")

OTHER PROFESSIONAL ACTIVITIES

2014 – 2015

- Member of BIUS, Association of Biology students, Faculty of Science, University of Zagreb

2012, 2013

- Participated in the event Night of Biology, won the Special Award from the Rector of the University of Zagreb for the participation in the 2012 event

July 2009

- **SEMEP** (South-Eastern Mediterranean Environmental Project) regional meeting of secondary school students, island of Vis, Croatia (presentation on the threat posed in the Adriatic by *Caulerpa Taxifolia*)

SKILLS

Microsoft Office tools
Sequence alignment algorithms
PHYLIP - phylogeny software package
Deep View (molecular graphics program)

Driving license B

HOBBIES

Playing the guitar; sports (martial arts, parkour, skiing)

LANGUAGES

Croatian – mother tongue; English – C1.2 certificate; German

Životopis

OSOBNE INFORMACIJE

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OBRAZOVANJE

od rujna 2014.	**Diplomski studij molekularne biologije** (apsolvent) Prirodoslovno- matematički fakultet Sveučilišta u Zagrebu
2011. – 2014.	**Preddiplomski studij molekularne biologije** Prirodoslovno- matematički fakultet Sveučilišta u Zagrebu; završen studij (192 ECTS) i stečeno zvanje *Sveučilišni prvostupnik (Baccalaureus) molekularne biologije* u rujnu 2014.
rujan 2007. – lipanj 2011.	Privatna klasična gimnazija, Zagreb; u lipnju 2011. položena državna matura iz šest predmeta (obavezni predmeti na višoj razini)

STRUČNO OSPOSOBLJAVANJE

ožujak - listopad 2016.

- Rad na **Europskom istraživačkom institutu za biologiju starenja (ERIBA)** u Groningenu, u laboratoriju izv. prof. dr.sc. **Michaela Changa** kao stipendist programa *Erasmus+*; provedeno istraživanje za diplomski rad pod naslovom **"Investigating the link between nonsense-mediated mRNA decay and survivor formation in *S. cerevisiae*"**

srpanj – kolovoz 2015.

- **Međunarodna ljetna škola (ISS)** na području regulacije gena, epigenetike i stabilnosti genoma: pohađanje predavanja te rad u laboratoriju dr. sc. **Petre Beli** na Institutu za Molekularnu biologiju u Mainzu na projektu **"Exploring the interaction between FEN1 and Cullin-RING ligases"**

srpanj 2014.

- Jednomjesečni boravak i rad u laboratoriju prof. dr.sc. **Klausa Förstemanna** u Centru za istraživanje gena (Gene Center) Sveučilišta Ludwig-Maximilian u Münchenu; upoznavanje s radom laboratorija i projektom prof. Förstemanna „Biogenesis and function of small RNAs" te izvođenje praktičnog rada u obliku mini-projekta

ožujak – svibanj 2014.

- Stručna praksa u laboratoriju izv. prof. dr. sc. **Ivane Ivančić Baće** na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu, u Zavodu za molekularnu biologiju, upoznavanje sa CRISPR/Cas sustavom na modelu *E. Coli*

srpanj 2013.

- 7-dnevni posjet laboratoriju prof. dr. sc**. Xaviera Perreta** u Zavodu za botaniku i biologiju biljaka Sveučilišta u Ženevi; stjecanje osnovnih laboratorijskih vještina te učenje o projektu prof. Perreta („Genetics of symbioses between *Sinorhizobium fredii* NGR234 and legumes"), praćenje rada drugih članova tima

srpanj 2012.

- 5-dnevni posjet laboratoriju prof. dr.sc. **Davida Shorea** u Zavodu za molekularnu biologiju Sveučilišta u Ženevi; praćenje i upoznavanje rada prof. Shorea („Establishing relationship between chromosome structure and the process of gene regulation, DNA replication, chromosome segregation"), praćenje rada drugih članova tima te stjecanje osnovnih laboratorijskih vještina uz njihovo mentorstvo

srpanj 2010.

- **Ljetna škola znanosti S3++** u Znanstveno-edukacijskom centru u Višnjanu; rad na grupnom projektu „Finding Antibiotic Resistance Genes Using Phyletic Profiling"

OSTALE STRUČNE AKTIVNOSTI

2014. – 2015.

- Član BIUS-a, Udruge studenata biologije na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu

2012., 2013.

- Sudjelovanje u manifestaciji „Noć biologije", što je 2012. god. nagrađeno Posebnom nagradom Rektora Sveučilišta u Zagrebu

srpanj 2009.

- Sudjelovanje na regionalnom skupu učenika srednjih škola **SEMEP** (South-Eastern Mediterranean Environmental Project) na otoku Visu, prezentacija o *Caulerpi Taxifolii* i opasnosti koju predstavlja za Jadransko more

VJEŠTINE

Računalne vještine: alati Microsoft Office™ , programi za sravnjivanje sekvenci, PHYLIP (phylogeny software package), program za molekularnu grafiku Deep View

Vozačka dozvola B kategorije

HOBIJI

Sviranje gitare
Sport (borilačke vještine, parkour, skijanje)

JEZICI
hrvatski (materinski)
engleski (svjedodžba za razinu C1.2)
njemački

APPENDIX I

Growth media and plates used in the study

YPD broth

- per 500 mL:
  - 5 g yeast extract
  - 10 g peptone
  - to 480 mL with demi H_2O
  - 20 mL of 50% glucose

YPGal broth

- per 500 mL:
  - 5 g yeast extract
  - 10 g peptone
  - to 450 mL with demi H_2O
  - 50 mL of 20% galactose

YPD plates

- per liter:
  - 10 g yeast extract
  - 20 g peptone
  - 20 g agar
  - 1 pellet of NaOH
  - to 960 mL with demi H_2O
  - 40 mL of 50% glucose

YPGal plates

- per liter:
  - 10 g yeast extract
  - 20 g peptone
  - 20 agar
  - 1 pellet of NaOH
  - to 900 mL with demi H_2O
  - 100 mL of 20% galactose

YPD+Cu plates

- per liter:
  - 10 g yeast extract
  - 20 g peptone

- 20 agar
- 1 pellet of NaOH
- to 999 mL with demi H_2O
- 1 ml of 100 mM CuSO4

YPD+G418 plates

- per liter:
  - 10 g yeast extract
  - 20 g peptone
  - 20 g agar
  - 1 pellet of NaOH
  - to 960 mL with demi H_2O
  - 40 mL of 50% glucose
  - 1 mL of 200 mg/mL G418

YPD+G418 plates contain antibiotic geneticin (G418), final concentration of which was 200 mg/L. G418 blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells. Only yeast cells that possess kanamycin-resistance marker (*kanMX*) can grow on media with geneticin.

YPD+NAT plates

- per liter:
  - 10 g yeast extract
  - 20 g peptone
  - 20 g agar
  - 1 pellet of NaOH
  - to 960 mL with demi H_2O
  - 40 mL of 50% glucose
  - 500 µL of 200 mg/mL clonNAT

YPD+NAT plates contain antibiotic nourseothricin (clonNAT) that blocks protein biosynthesis. Final concertation of this antibiotic was 100 mg/L. Yeast cells that can grow on these plates contain nourseothricin-resistance marker (*natMX*) that confers resistance to the antibiotic.

Minimal (SD) media plates

- per liter:
  - 1.3 g dropout mix*
  - 6.7 g yeast nitrogen base without amino acids
  - 20 g agar

    - 1 pellet of NaOH
    - to 960 mL with demi H_2O
    - 40 mL of 50% glucose
- *dropout mix:
    - for SD-his plates, –his dropout mix was used
    - for SD-ura plates, –ura dropout mix was used

Nutrient	Amount in dropout mix (g)
Adenine (hemisulfate salt)	2.5
L-arginine (HCl)	1.2
L-aspartic acid	6.0
L-glutamic acid (monosodium salt)	6.0
L-histidine	1.2
L-leucine	3.6
L-lysine (mono-HCl)	1.8
L-methionine	1.2
L-phenylalanine	3.0
L-serine	22.5
L-threonine	12.0
L-tryptophan	2.4
L-tyrosine	1.8
L-valine	9.0
Uracil	1.2

- –his dropout mix lacks histidine but contains the other nutrients listed in the table.
- –ura dropout mix lacks uracil but contains the other nutrients listed in the table.

SD-URA plates and SD-HIS plates are minimal media plates that are prepared without uracil or histidine. Yeast cells that grow on SD-URA plates contain URA3 marker and the ones that can grow on SD-HIS plates have HIS3 marker. URA3 is a gene located on chromosome V. *URA3* encodes orotidine-5'phosphate decarboxylase, an enzyme required for the biosynthesis of uracil. *HIS3* is a gene located on chromosome XV. This gene codes for imidazoleglycerol-phosphate dehydratase, an enzyme that catalyzes the sixth step in histidine biosynthesis.

minSPO plates

- per litre:
    - 10 g KOAc
    - 50 mg ZnOAc
    - 20 agar (washed 3x with demiH_2O)
    - 1 pellet of NaOH
    - to 1 L with demi H_2O
    - 2.5 mL of SPO supplement (kept at 4°C)

APPENDIX II

Solutions used for Li/Ac transformation

10x TE

- 100 mM Tris-Cl (pH=7.4)
- 10 mM EDTA

10x LiAc

- 1 M LiAc

1x TE/LiAc

- 10x TE
- 10x LiAc
- dH2O

40% PEG4000

- 10x TE
- 10x LiAc
- 50% PEG4000

APPENDIX III

Solutions used for Southern blot

20x SSC (pH=7)

- 3 M NaCl
- 0.34 M $C_6H_5Na_3O_7$

5x DIG wash buffer (pH=7.5)

- 0.5 M Maleic acid
- 0.75 M NaCl
- 1.5% Tween 20

Maleic acid buffer (pH=7.5)

- 0.1 M Maleic acid
- 0.15 M NaCl

DIG detection buffer (pH=9.5)

- 0.1 M Tris-HCl
- 0.1 M NaCl

Denaturing solution (pH>>10)

- 1.5 M NaCl
- 0.5 M NaOH

Neutralization solution (pH=7.5)

- 0.5 M Tris-HCl
- 1 M NaCl

10x Blocking solution

- Blocking reagent (Roche) diluted in Maleic acid buffer