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Molecular Mechanism for Degradation of Transcriptionally Stalled RNA Polymerase II in the Yeast *Saccharomyces cerevisiae*

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ERKLÄRUNG

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EHRENWÖRTLICHE VERSICHERUNG

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SUMMARY

Transcription of protein coding genes by RNA polymerase II (RNAPII) is an essential step in gene expression. Transcription elongation is a highly dynamic and discontinuous process that includes frequent pausing of RNAPII, backtracking, and arrest both *in vitro* and *in vivo*. Consequently, a multitude of transcription elongation factors are needed for efficient transcription elongation. When transcription elongation factors fail to "restart" RNAPII the persistently stalled RNAPII complex prevents transcription and thus has to be recognized and removed to free the gene for subsequent polymerases. Similarly, DNA damage causes stalling of RNAPII. In this case, the DNA damage is either repaired by Transcription-Coupled Repair (TCR) or RNAPII is degraded as a "last resort" mechanism by the ubiquitin proteasome system. In contrast to RNAPII degradation caused by DNA damage, the cellular pathway for removal of transcriptionally stalled RNAPII complexes has remained largely obscure. However, it was speculated that transcriptionally stalled RNAPII complexes are degraded by the same pathway as RNAPII stalled due to DNA damage. Here, it is shown that the pathway for degradation of transcriptionally stalled RNAPII is distinct from the DNA damage-dependent pathway, providing the first evidence that the cell distinguishes between RNAPII complexes stalled for different reasons. The novel cellular pathway for transcriptional stalling-dependent degradation of RNAPII is termed TRADE. Specifically, in the TRADE pathway a different yet overlapping set of enzymes is responsible for poly- and de-ubiquitylation of transcriptionally stalled RNAPII. Moreover, the catalytic 20S proteasome is recruited to transcribed genes indicating that Rpb1 of transcriptionally stalled RNAPII complexes is degraded at the site of transcription. Importantly, nucleotide starvation and temperature stress which might mimic natural conditions of transcription elongation impairment also lead to RNAPII degradation. Finally, this study provides the first evidence that the mechanism for the controlled degradation of the transcriptionally stalled RNA polymerase complex might also exist for transcription by RNAPI and RNAPIII. Taken together, the TRADE pathway elucidated in this study ensures continued transcription.

PUBLICATIONS

Parts of the present thesis are submitted for publication:

Karakasili, E. and Sträßer, K. "A novel pathway for degradation of transcriptionally stalled RNA polymerase II", under revision

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1. INTRODUCTION

1.1. THE CENTRAL DOGMA OF MOLECULAR BIOLOGY

Gene expression is a fundamental cellular process through which a certain genotype results in the corresponding phenotype. In 1958 Francis Crick introduced the Central Dogma of Molecular Biology as the concept behind gene expression (Crick, 1970; Thieffry and Sarkar, 1998). The central dogma describes how the information-containing deoxyribonucleic acid (DNA) is transcribed to the intermediate ribonucleic acid (RNA) which in turn becomes translated into proteins (Figure 1).

Figure 1| The Central Dogma of Molecular Biology.

The transcription from DNA to RNA is mediated by multiprotein complexes termed DNA-dependent RNA polymerases (RNAP). However, in 1970 it was discovered that reverse transcription can also take place in a reaction mediated by RNA-dependent DNA polymerases also known as reverse transcriptases (Baltimore 1970; Temin and Mizutani 1970). Moreover, around the same time RNA replication was reported for RNA-viruses (Penhoet, Miller et al. 1971; Skehel 1971). The final step of gene expression for protein coding genes is the translation of the intermediate molecule RNA into proteins the molecules responsible for the phenotype. Translation is mediated by the ribosome, a large molecular weight complex made from RNAs and proteins.

1.2. DNA-DEPENDENT RNA POLYMERASES

Transcription in eukaryotes is performed by three DNA-dependent RNA polymerases (RNAPs), which are functionally and structurally related (Cramer, Armache et al. 2008). Each of them is a multisubunit complex responsible for the synthesis of different classes of RNA. Table 1 summarizes their subunit composition as well as the subunits shared in the yeast *Saccharomyces cerevisiae* (Archambault and Friesen 1993; Akira, Makoto et al. 1998).

Table 1| Subunit composition including subunits shared between *S.cærevisiae* RNA polymerases. Modified from (Cramer, Armache et al. 2008). Subunits which are unique to its enzyme are colored accordingly.

DNA-dependent RNA polymerase	RNAPI	RNAPII	RNAPIII
Enzyme Core	Rpa190 Rpa135 Rpc40 (AC40) Rpa12 Rpc19 (AC19) Rpb5 (ABC27) Rpb6 (ABC23) Rpb8 (ABC14,5) Rpb10 (ABC10α) Rpb12 (ABC10β)	Rpb1 Rpb2 Rpb3 Rpb9 Rpb11 Rpb5 (ABC27) Rpb6 (ABC23) Rpb8 (ABC14,5) Rpb10 (ABC10α) Rpb12 (ABC10β)	Rpc160 Rpc128 Rpc40 (AC40) Rpc11 Rpc19 (AC19) Rpb5 (ABC27) Rpb6 (ABC23) Rpb8 (ABC14,5) Rpb10 (ABC10α) Rpb12 (ABC10β)
Other subunits	Rpa49 Rpa43 Rpa34 Rpa14	Rpb7 Rpb4	Rpc82 Rpc53 Rpc37 Rpc34 Rpc31 Rpc25 Rpc17
Total number of subunits	14	12	17

RNA polymerase II (RNAPII) mediates transcription of protein-coding genes and many noncoding RNAs, including all spliceosomal small nuclear RNAs (snRNAs) except U6, small nucleolar RNAs (snoRNAs), microRNA (miRNA) precursors, and cryptic unstable transcripts (CUTs). RNA polymerase I (RNAPI) transcribes the abundant ribosomal RNAs (rRNAs), and RNA polymerase III (RNAPIII) transcribes noncoding RNAs such

as transfer RNAs (tRNAs), 5S rRNA, and U6 spliceosomal snRNA. Recently, two additional types of RNAP were discovered in plants. These two RNAPII-related, plant-specific enzymes, named RNAPIV and V, collaborate with proteins of the RNA interference machinery to generate long and short noncoding RNAs involved in epigenetic regulation (Till and Ladurner 2007; Matzke, Kanno et al. 2009).

1.3. Modification of the Carboxyl-Terminal Domain (CTD) of RNAPII

RNAPII is responsible for the transcription of all mRNA encoding genes. A unique feature of RNAPII that sets it apart from the other polymerases is the extended carboxyl-terminal domain (CTD) of its largest subunit, Rpb1. The CTD of Rpb1 consists of a varying number of tandemly repeated heptapeptides with the consensus sequence Y^1-S^2-P^3-T^4-S^5-P^6-S^7 (Stiller and Hall 2002; Svejstrup 2004). The consensus repeat has been conserved in evolution although the number of repeats varies between different species. RNAPII of mammalian cells contains 52 copies of the consensus repeat, and *S.cerevisiae* contains 26–27 copies, whereas other eukaryotes contain an intermediate number of repeats (Stiller and Hall 2002). The CTD has modification-specific protein interactions through which RNAPII proceeds in the transcription cycle (see below). The CTD modifications include phosphorylation (mostly on Ser2 and Ser5), glycozylation, and *cis/trans* isomerization of prolines (Figure 2) (reviewed in (Lin, Tremeau-Bravard et al. 2003)).

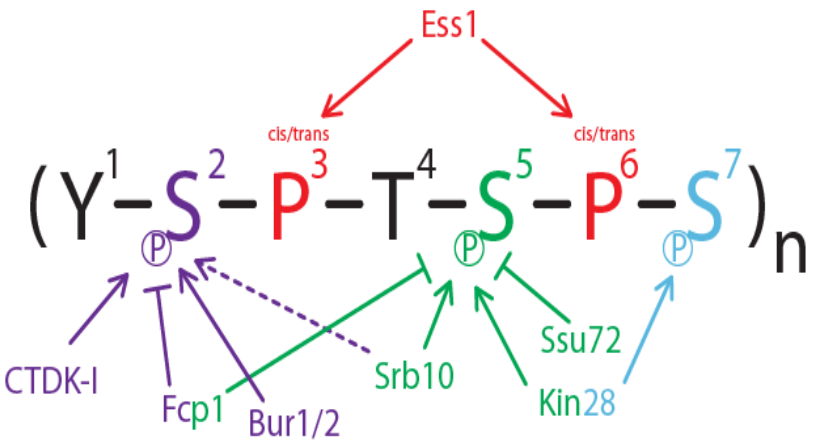

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Figure 2| The CTD modifications. Most of the residues in the heptapeptide repeat of the CTD are subject to modification. Phosphorylation of the three serine residues and isomerization of the two proline residues are the main CTD modifications. Enzymes are colored according to their involvement in the modification of a specific site.

Several proteins have been shown to regulate the phosphorylation status of the CTD (Bensaude, Bonnet et al. 1999). In *S.cerevisiae* Srb10 and Kin28 (CDK7 and CDK8 in mammals) phosphorylate the CTD at Ser5. Srb10 is also responsible for phosphorylating Ser2 (Hengartner, Myer et al. 1998; Sun, Zhang et al. 1998). The CTDK-I complex and the Bur1/2 complex (CDK9 in mammals) predominantly phosphorylate the CTD at Ser2 (Sterner, Lee et al. 1995; Cho, Kobor et al. 2001; Murray, Udupa et al. 2001). In *S.cerevisiae*, Ssu72 (SCP1 in mammals) is a Ser5 phosphatase and has been linked to all stages of transcription, from initiation to elongation and termination (Kim, Vasiljeva et al. 2006; Reyes-Reyes and Hampsey 2007). Another phosphatase, Fcp1 dephosphorylates both Ser2 and Ser5 *in vitro* (Cho, Kim et al. 1999; Lin, Marshall et al. 2002), although there are discrepancies as to whether there is preference towards one of these two sites (Sims, Belotserkovskaya et al. 2004). Upon termination, Fcp1 can recycle RNAPII molecules for new rounds of transcription (Cho, Kim et al. 1999).

Furthermore, the CTD is also glycozylated by beta-O-linked GlcNAc on a subset of RNAPII molecules. Initially, it was hypothesized that the reason for this modification is the regulation of phosphorylation by sterically blocking sites of kinase action (Kelly, Dahmus et al. 1993). However, it was later discovered that the enzymes responsible for the glycozylation and phosphorylation of the CTD are mutually exclusive, suggesting that *in vivo* this could result in their differential association with the CTD at specific stages during the transcription cycle (Comer and Hart 2001).

Finally, the phosphorylation-specific peptidyl-prolyl cis-trans isomerise Ess1 (PIN1 in mammals) was shown to regulate RNAPII transcription by altering the cis-trans ratio of proline-containing peptide bonds in the CTD (Verdecia, Bowman et al. 2000; Wu, Rossettini et al. 2003; Xu and Manley 2004)

Taken together, modification of the CTD most likely influences its conformation and thus its association with factors which function at different stages of transcription. Additionally, CTD modifications influence the recruitment of the mRNA processing machinery for capping, splicing, and polyadenylation of the message, to produce an export competent messenger ribonucleoprotein particle (mRNP) (reviewed in (Hirose and Ohkuma 2007)) . So there seems to be a "CTD code" that creates a differential scaffold for the enzymes that catalyse the next step of transcription. This code could ensure (i) the successful synchronization of the RNA processing reactions so that they occur in the correct order, (ii) the efficient transitions between the reactions, and (iii) the assurance that no step is omitted (Dahmus 1996; Orphanides and Reinberg 2002). Different CTD modifications are characteristic of the different transcription stages and are hence important for the progression through the transcription cycle.

1.4. THE TRANSCRIPTION CYCLE

Gene transcription by RNAPII consists of different stages, whose recycling make up the transcription cycle (Figure 3). There are three main parts in the cycle, known as initiation, elongation and termination, and each of them is regulated by transcription factors (Bregman, Pestell et al. 2000; Panning and Taatjes 2008).

During transcription initiation, non-phosphorylated RNAPII assembles at the core promoter together with several general transcription factors (GTFs) and coactivators (such as the Mediator) to form the preinitiation complex (PIC) (Bushnell, Westover et al. 2004; Rani, Ranish et al. 2004; Chen, Warfield et al. 2007). In the PIC the dsDNA of the promoter is melted and thus becomes accessible to the polymerase. This results in the formation of a transcription bubble, *i.e*. the open complex (Brueckner, Ortiz et al. 2009). During transcription initiation the CTD is phosphorylated on Ser5 (Cho, Kobor et al. 2001). It was shown that early in initiation the polymerase can produce abortive transcripts. These abortive cycles have been observed with a single RNAP releasing several transcripts without escaping the promoter (Venters and Pugh 2009).

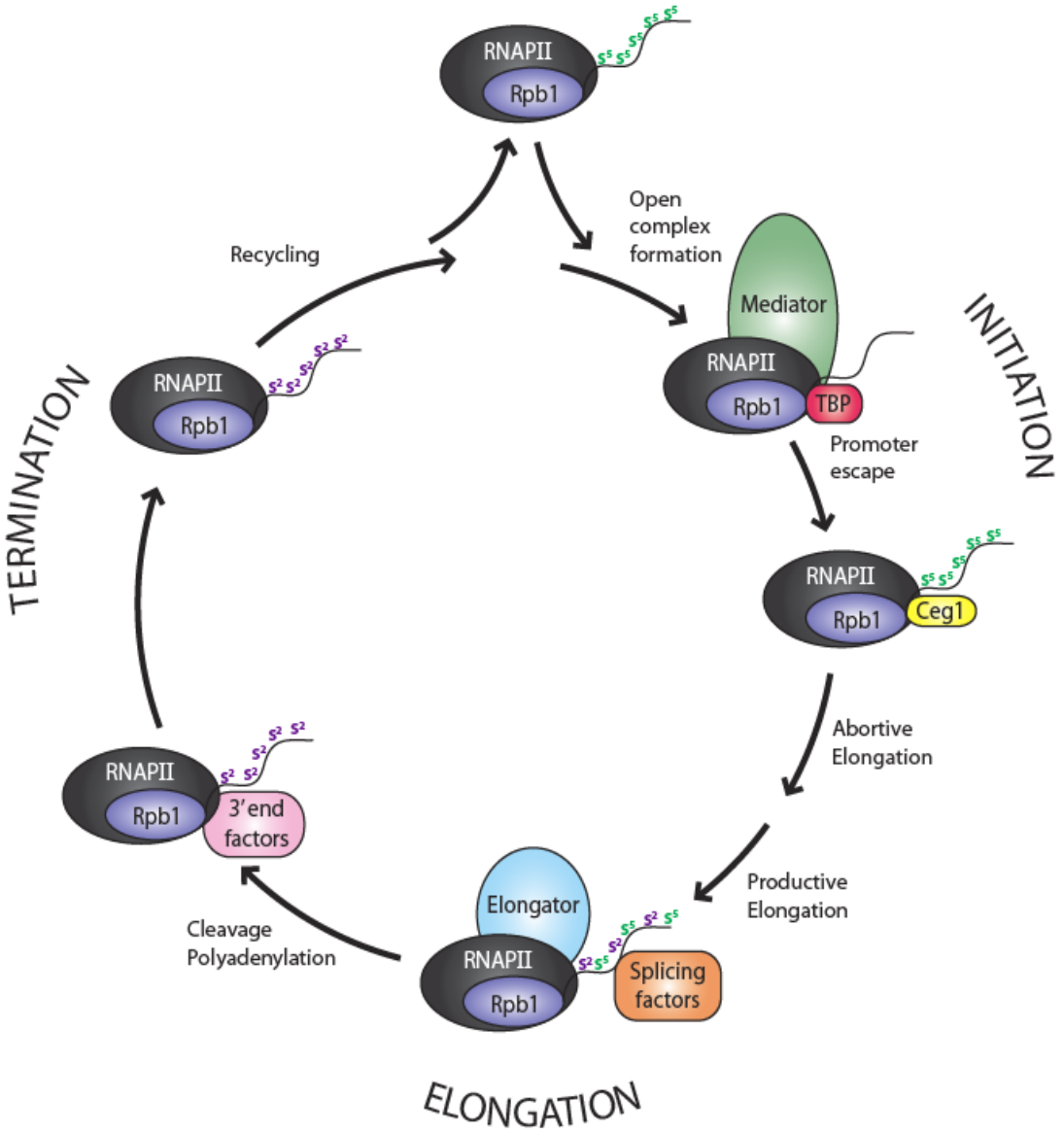

Figure 3| The mRNA transcription cycle. There are three main parts in the cycle: initiation, elongation and termination. The transcribing complex, mostly through the CTD modifications, associates with different factors in the different stages. At the end of each cycle RNAPII is recycled and can enter a new round of transcription.

RNAPII can then initiate template DNA-dependent mRNA synthesis. In the active site nucleotides are paired with the template and joined processively during elongation to produce the RNA transcript (Cramer, Bushnell et al. 2001). At the transition from promoter escape to processive transcription elongation, the levels of Ser5 phosphorylation are reduced and the CTD becomes mainly phosphorylated on Ser2 (Komarnitsky, Cho

et al. 2000). Termination of transcription involves the release of the RNA transcript and the dissociation of RNAPII from the DNA template (Richard and Manley 2009). Several studies showed that RNAPII termination is coupled to 3'-end processing of the pre-mRNA (reviewed in (Buratowski 2005). The released RNAPII is then recycled and is able to enter a new round of transcription.

1.5. TRANSCRIPTION IS A HIGHLY DYNAMIC AND DISCONTINUOUS PROCESS

Transcription is a fundamental step in gene expression and ensuring its continuation is important for the survival of the cell. Hence, it is one of the most regulated and complex processes requiring the function of numerous auxiliary factors (Arndt and Kane 2003; Hirose and Ohkuma 2007; Fuda, Ardehali et al. 2009). Most studies on transcriptional regulation have focused on promoter regulation and transcription initiation. Promoter-proximal pausing is the phenomenon where RNAPII pauses at the 5' region of the open reading frame (ORF). It can then progress efficiently into productive elongation only in response to appropriate signals. Promoter-proximal pausing functions as a checkpoint before committing to productive elongation. Importantly, even though RNAPII can escape rapidly from the pause (Lis 1998) this escape continues to be a rate-limiting and regulatory step after gene induction (Giardina, Perez-Riba et al. 1992).

However, there are more and more studies emerging suggesting that events subsequent to initiation could be tightly regulated. To that end, as demonstrated by *in vitro* studies with prokaryotic polymerases, the elongation step can be regulated by pausing for various times (Davenport, Wuite et al. 2000; Tadigotla, O'Maoileidigh et al. 2006). For eukaryotic cells, several studies attempted to shed light on the transcription rate of RNAPII. These included radioactive quantitative northern blot analysis (Thummel, Burtis et al. 1990), nuclear run-on assays (O'Brien and Lis 1993), chromatin immunoprecipitation (ChIP) of RNAPII on inducible genes (Mason and Struhl 2005), quantitative reverse transcription PCR (q-RT PCR) following induction of expression (Tennyson, Klamut et al. 1995), and *in situ* visualization of specific mRNAs (Femino,

Fay et al. 1998). In these studies, elongation was thought to be continuous with a calculated rate from 1,1-2,4 kb/min. However, recent studies in living mammalian cells gave new insights into the *in vivo* kinetics of RNAPII transcription and introduced the concept of frequent RNAPII pausing instead of continuous RNAPII transcription (Figure 4) (Darzacq, Shav-Tal et al. 2007; Pelechano, Jimeno-Gonzalez et al. 2009; Singh and Padgett 2009).

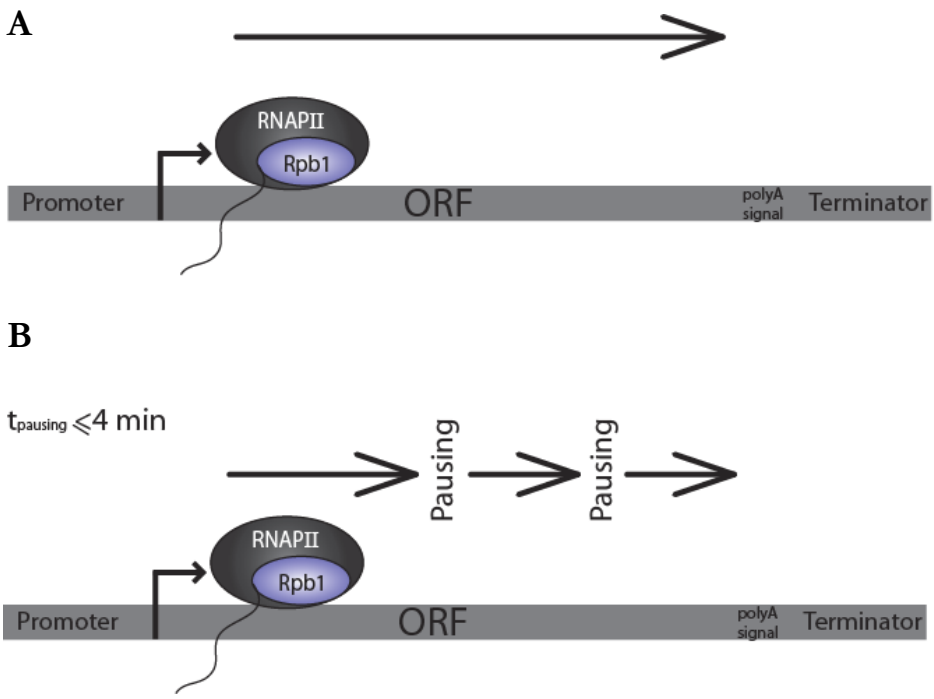

Figure 4| Schematic representation of RNAPII pausing during transcription.
RNAPII transcription was considered to be a continuous process (**A**). However, recent data showed that transcription is discontinuous (**B**) as it involves frequent pausing of the transcribing RNAPII across the ORF with a calculated maximum time of four minutes.

Interestingly, it was shown that only a minor fraction of the polymerases assembling at the promoter ultimately produce a transcript. More importantly in about 4% of these polymerases that enter elongation there is frequent pausing and arrest of RNAPII. Based on the experimental data, RNAPII elongation was divided into two subclasses: rapid elongation occurring at a speed of 4,3kb/min (up to four times faster than once thought)

and probabilistic pausing that can last for up to four minutes. However, due to technical limitations it could not be distinguished whether a single polymerase was engaging in multiple short pauses or in one long arrest. Furthermore, another study that applied the same methodology found that transcription by RNAPI is also rather inefficient (Dundr, Hoffmann-Rohrer et al. 2002).

A number of proteins facilitate paused RNAPII complexes to resume transcription (Awrey, Weilbaecher et al. 1997; Nesser, Peterson et al. 2006; Ardehali and Lis 2009). Nevertheless, it was suggested that the frequent pausing of RNAPII could lead to its irreversible arrest (Svejstrup 2007; Margaritis and Holstege 2008; Daulny and Tansey 2009), an event that would prevent continuation of transcription and subsequently cell viability.

1.6. TRANSCRIPTION ELONGATION FACTORS

Transcription elongation by RNAPII is regulated at different stages by auxiliary protein factors (Saunders, Core et al. 2006). There are three main categories of transcription elongation factors. The first, a typical example of which is TFIIS, re-activates elongation complexes that have paused or arrested during elongation (Wind and Reines 2000). The second class, which includes among others the CTDK-I and the Bur1/2 kinase complexes, facilitates escape of the elongation complex from the promoter (Cho, Kobor et al. 2001; Jona, Wittschieben et al. 2001; Keogh, Podolny et al. 2003). Finally, in the third class, proteins such as Spt4/Spt5 and the THO complex have been shown to increase the general efficiency of elongation (Hartzog, Wada et al. 1998; Jimeno, Rondon et al. 2002; Strasser, Masuda et al. 2002; Rondon, Jimeno et al. 2003).

1.6.1. The CTDK-I kinase complex

The CTDK-I complex is composed of three subunits, Ctk1, Ctk2, and Ctk3. Ctk1, a cyclin-dependent kinase, is the catalytic subunit, Ctk2 is the cyclin, while Ctk3 shows no

similarity to other known proteins (Sterner, Lee et al. 1995). The assembly of those three subunits leads to an active protein kinase complex that phosphorylates the CTD of RNAPII on Ser2 of the heptapeptide YSPTSPS repetitive sequence (see Introduction 1.3). Ctk1 is suggested to be the primary Ser2 kinase during transcription elongation (Cho, Kobor et al. 2001). Even though the *CTK1* gene is not essential its deletion results in a severe growth defect (Ostapenko and Solomon 2005).

The CTDK-I complex is involved in multiple steps of transcription. Ctk1 plays a role in (i) the release of basal transcription factors from RNAPII as it enters productive elongation (Ahn, Keogh et al. 2009), (ii) coupling transcription elongation to 3' end processing and transcription termination (Ahn, Kim et al. 2004; Ni, Schwartz et al. 2004), (iii) splicing (Morris and Greenleaf 2000), (iv) methylation of histones (Xiao, Shibata et al. 2007) and (v) DNA damage-induced transcription (Ostapenko and Solomon 2003). Furthermore, Ctk1 is most likely involved in the control of transcription elongation since it modulates the elongation efficiency of RNAPII in mammalian HeLa extracts (Lee and Greenleaf 1997) and interacts genetically with various factors that control transcription elongation (Jona, Wittschieben et al. 2001; Hurt, Luo et al. 2004).

Surprisingly, Ctk1 was also found to play a role in RNAPI transcription (Bouchoux, Hautbergue et al. 2004) and in translation elongation by phosphorylation of the ribosomal protein Rps2 (Rother and Strasser 2007).

1.6.2. The elongation cleavage factor TFIIS

The elongation factor TFIIS (also called Dst1) is an RNA cleavage stimulatory factor that enables RNAPII to escape arrest during transcription elongation. Dst1 binds to the stalled complex and stimulates RNAPII's intrinsic cleavage activity for the nascent RNA. This cleavage allows RNAPII to resume transcription by placing the 3' end of the transcript in the active site of the enzyme (Wind and Reines 2000). By structural studies it became evident that the C-terminus of Dst1 is able to reach deeply into the RNAPII secondary channel, approaching the catalytic site of the polymerase (Kettenberger, Armache et al. 2003). This proximity between Dst1 and the nascent RNA may trigger

the endogenous nuclease activity of RNAPII, which permits the stalled complex to restart the elongation (Prather, Larschan et al. 2005).

Mutations in the *DST1* gene cause sensitivity to 6-Azauracil (6AU) (Mason and Struhl 2005), a drug that depletes cellular GTP/UTP levels and is thus used as a phenotypic marker for transcription elongation impairment (Exinger and Lacroute 1992). Moreover, *DST1* genetically interacts with various components of the transcription elongation machinery such as Spt4/Spt5 (Wind and Reines 2000; Lindstrom and Hartzog 2001) and references therein). Furthermore, upon stress conditions such as low temperature, heat shock, or the presence of 6AU in the growth medium, Dst1 was found to be recruited to the ORF of several genes (Pokholok, Hannett et al. 2002). In addition to its role in transcription elongation, some studies have indicated that Dst1 may also play a role in transcription initiation (Davie and Kane 2000; Malagon, Tong et al. 2004; Guglielmi, Soutourina et al. 2007). Finally, Dst1 was recently found to play a role in RNAPIII transcription (Ghavi-Helm, Michaut et al. 2008).

1.6.3. The THO complex

The THO complex is a nuclear protein complex conserved from yeast to human that is involved in the biogenesis of mRNP particles which are then exported from the nucleus (reviewed in (Jimeno and Aguilera 2010). In *S.cerevisiae*, the RNAPII-associated THO complex consists of the four proteins Mft1, Hpr1, Tho2 and Thp2. None of these THO components are essential for cell viability. However, deletion of each of these four genes results in similar phenotypes indicating that THO is a functional and physical unit. Specifically, it was shown that the THO complex is important for the formation of the correct mRNP particle since it prevents the nascent RNA from interacting with the DNA template (Huertas and Aguilera 2003). Such an interaction would lead to the formation of a DNA-RNA hybrid, also known as R-loop, which would prevent elongation by the next polymerase (Huertas, Garcia-Rubio et al. 2006). The formation of R-loops has been linked to hyper-recombination, a phenotype observed in all the deletion mutants of THO (Chavez, Beilharz et al. 2000; Jimeno, Rondon et al. 2002). Additionally, the

THO complex is present at actively transcribed genes over the entire length of the ORF (Strasser, Masuda et al. 2002; Zenklusen, Vinciguerra et al. 2002; Kim, Ahn et al. 2004) and is needed for efficient transcription elongation (Chavez and Aguilera 1997; Piruat and Aguilera 1998; Chavez, Beilharz et al. 2000; Strasser, Masuda et al. 2002; Rondon, Jimeno et al. 2003). Moreover, several studies showed that the THO complex is a conserved nuclear factor with a key function in mRNP biogenesis and export (Strasser, Masuda et al. 2002; Zenklusen, Vinciguerra et al. 2002; Huertas, Garcia-Rubio et al. 2006; Rougemaille, Dieppois et al. 2008) as well as in development and cell differentiation (Wang, Chang et al. 2006; Wang, Chinnam et al. 2009). For its function in mRNA export, the THO complex interacts with Sub2 and Yra1 (mRNA export factors), Gbp2 and Hrb1 (SR-like proteins) and Tex1 to form the so called TREX complex that couples TRanscription to mRNA EXport (Strasser and Hurt 2000; Strasser and Hurt 2001).

1.6.4. The Bur1/Bur2 kinase complex

The BUR kinase complex, which consists of the cyclin-dependent kinase Bur1, a protein essential for viability, and the corresponding cyclin Bur2 (Yao, Neiman et al. 2000) was shown to be important for efficient transcription elongation (Keogh, Podolny et al. 2003). Deletion of *BUR2* results in an impaired growth phenotype. The Bur1 kinase phosphorylates the CTD of RNAPII *in vitro* (Murray, Udupa et al. 2001) and *in vivo* (Qiu, Hu et al. 2009). Furthermore, Bur1 binds to initiating RNAPII since it copurifies with the Ser5 phosphorylated form of the enzyme (Lindstrom and Hartzog 2001). As another *in vitro* substrate of Bur1 the E2 ubiquitin-conjugating enzyme Rad6 was identified. Subsequently, the Rad6 phosphorylation leads to the ubiquitylation of histone H2B on K123 (Wood, Schneider et al. 2005) which in turn is required for H3K4 methylation (a histone modification present at actively transcribed genes) by the COMPASS complex (Wood, Schneider et al. 2003; Wood, Schneider et al. 2005; Lee, Shukla et al. 2007). Finally, a recent study identified the protein Spt5 as an *in vivo* substrate of the Bur1/2 kinase complex (Zhou, Kuo et al. 2009). Based on this finding

and since Spt5 promotes RNAPII transcription elongation (Hartzog, Wada et al. 1998) it was proposed that the effect of Bur1/2 in transcription elongation is partially mediated by the phosphorylation of Spt5 in its C-terminal region (CTR) (Zhou, Kuo et al. 2009). Furthermore, combining the deletion of the CTR of Spt5 with a C-terminal TAP-tag on *RAD6* (the other Bur1 substrate) resulted in a severe growth phenotype. This synthetic sickness phenotype suggests that the requirement of the *BUR1* gene for cell viability could be due to the fact that the kinase phosphorylates both Rad6 and the Spt5 (Zhou, Kuo et al. 2009).

1.7. THE UBIQUITIN-PROTEASOME PATHWAY (UPP)

The degradation of a protein is mediated through two distinct pathways. The first one involves the degradation of extracellular proteins that are taken up by the cell via endocytosis and are then transferred through a series of vesicles to primary lysosomes where they are degraded. This kind of degradation is not specific and all proteins that are taken up by the lysosomes are degraded at more or less the same rate (reviewed in (Todde, Veenhuis et al. 2009). The observation that the different intracellular proteins have different half-lives led to the prediction that there is another pathway for protein degradation, one that should have a high degree of specificity towards its substrate. The finding of the UPP verified this prediction (Etlinger and Goldberg 1977; Ciehanover, Hod et al. 1978).

Protein degradation by the UPP comprises two successive steps. First, a polyubiquitin chain that consists of covalently linked ubiquitin molecules is attached to the target. Second, the polyubiquitylated substrate is degraded by the 26S proteasome complex in a reaction that recycles the ubiquitin molecules. A schematic representation of the pathway is shown in Figure 5.

1.7.1. Polyubiquitylation of the substrate

Ubiquitin is a 76 amino acid globular protein that is highly conserved throughout evolution. The attachment of the polyubiquitin chain to the substrate is a three-step process (Figure 5, left pannel) (Pickart 2004). The first step is an ATP-required reaction mediated by the ubiquitin-activating enzyme also known as E1. During this reaction the E1-S~intermediate is generated which contains a high-energy thiol-ester bond (S). Then one of the several ubiquitin-conjugating enzymes, called E2s, generates a high-energy thiol-ester E2-S~ubiquitin intermediate. The third step depends on the type of the ubiquitin-protein ligase E3 that is used for each specific substrate. There are two main types of E3 ligases (Ardley and Robinson 2005). In the first type the E3 of the RING-finger/U-box family recognizes the substrate to allow ubiquitin transfer directly from the

E2. In the second type the E3 of the HECT domain E3 family recognizes the substrate directly and ubiquitylates it. Here, the ubiquitin transfer is from the E2 to the E3 and then to the substrate. In the case of the HECT E3s, a third high-energy thiol ester E3-S~ubiquitin intermediate is generated, before the activated ubiquitin molecule is transferred to the E3-bound substrate. In any case, the final step is the covalent conjugation of ubiquitin to the substrate by the E3 enzyme.

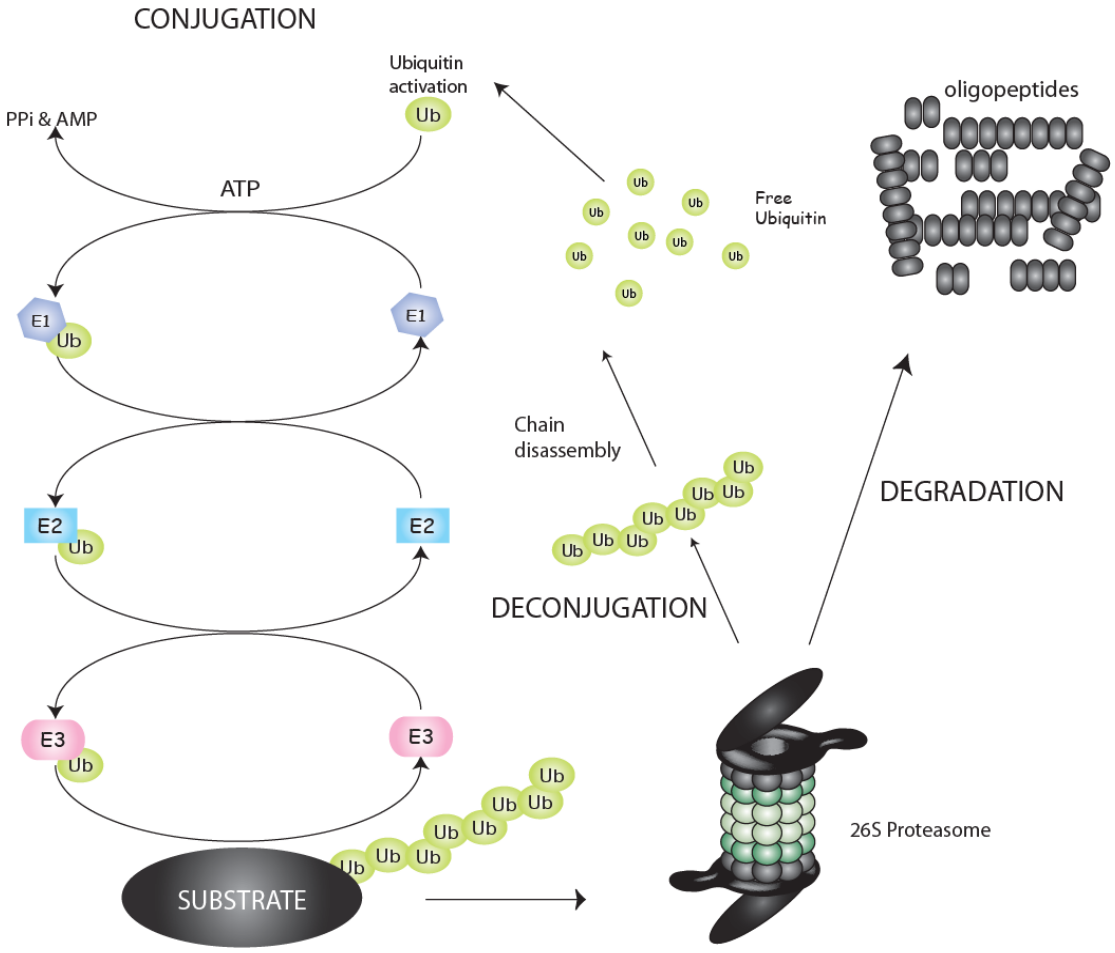

Figure 5| Schematic representation of the UPP. Degradation of a protein by the UPP is divided into two main steps; polyubiquitylation of the substrate (left panel) followed by its proteasome-mediated degradation (right panel.). See text for details

Successive steps of this reaction result in the further addition of ubiquitin molecules on the substrate. A polyubiquitin chain is then formed and each ubiquitin is linked with the previous and the next molecule via an internal lysine residue of ubiquitin (reviewed in (Hochstrasser 1996; Hershko and Ciechanover 1998; Hochstrasser, Johnson et al. 1999;

Glickman and Ciechanover 2002). In *S.cerevisiae*, all seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) of ubiquitin can be used for chain formation, resulting in chains of different topologies. These lysines are used in the frequency order of K48>K63 & K11>>K33, K27, K29 and K6 (Peng, Schwartz et al. 2003). Of these chains, the best understood type is the polyubiquitin chain linked through K48 of ubiquitin. K48-linked chains with a length of four or more ubiquitins serve as the predominant proteasome-targeting signal (Thrower, Hoffman et al. 2000; Pickart and Fushman 2004). K11- and K29-linked chains are also involved in proteasome dependent protein degradation (Baboshina and Haas 1996; Koegl, Hoppe et al. 1999; Jin, Williamson et al. 2008). In contrast, K63-linked chains and mono-ubiquitylation are generally thought to function in proteasome-independent processes such as DNA repair, signal transduction and receptor endocytosis *in vivo* (Hicke 2001; Pickart and Fushman 2004). However, a number of *in vitro* (Hofmann and Pickart 2001; Lee and Sharp 2004; Kim, Kim et al. 2007) and *in vivo* studies (Saeki, Kudo et al. 2009) suggest that K63-linked chains also support the proteasomal degradation of the substrate. Much less is known about the functions of chains with other topologies (Figure 6). The polyubiquitin chain is then recognised by the downstream 26S proteasome complex.

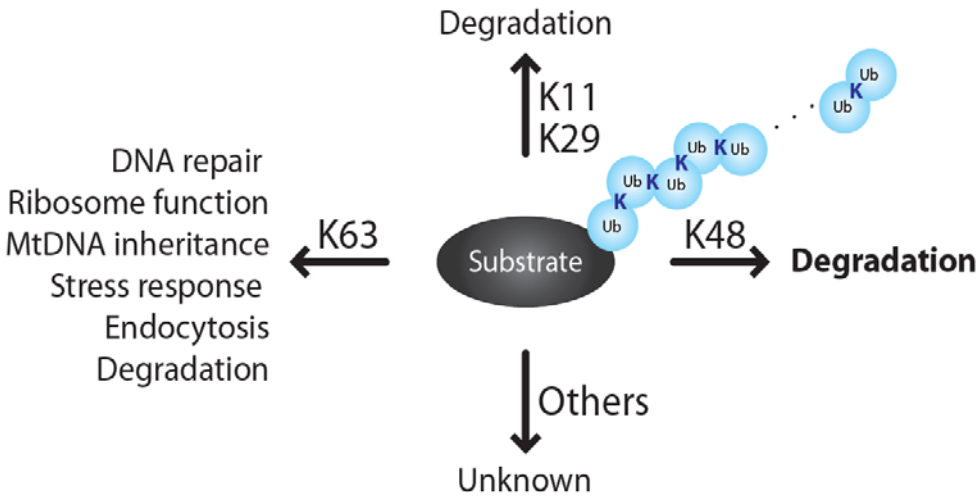

Figure 6| Lysine-specific ubiquitin chains serve different functions. Different lysine residues of ubiquitin can be used for chain formation, resulting in chains of different topologies. K48 and K63 are the two mostly used lysines. K48-specific chains serve mainly for degradation while K63-specific chains serve mainly for regulatory purposes as well as degradation. See text for details

1.7.2. Degradation by the 26S Proteasome

The proteasome is a multicatalytic protease composed of a 20S core particle, which catalyses the degradation of polyubiquitylated proteins into small peptides, and two 19S regulatory particles (Figure 7). The catalytic component of the proteasome, the 20S particle, is a cylindrical chamber of 28 subunits that form two identical inner β-rings and two identical outer α-rings (Figure 7, green subunits) (reviewed in (Hanna and Finley 2007; Tanaka 2009). The general structure is $\alpha_7\beta_7\alpha_7\beta_7$. Three of the β-subunits –β1, β2 and β5– contain the six active sites of the particle. The active site is a single threonine residue that is located at the amino-terminus of the β subunits (Groll, Ditzel et al. 1997; Unno, Mizushima et al. 2002). The 20S core is capped at each end by a 19S particle. This particle has two multisubunit components: the "base", which is composed of six ATPases and two non-ATPases, and the "lid", which includes 8 non-ATPase subunits (Figure 7, black subunits) (Glickman, Rubin et al. 1998). The functions of the 19S component include: (i) recognition of polyubiquitylated proteins, (ii) formation of an opening in the α-ring by which the entry of the target protein into the proteolytic chamber is allowed, and (iii) presumably unfolding of the substrate and its introduction into the catalytic particle. The six different ATPase subunits provide the energy required for most of the 19S functions (Glickman, Rubin et al. 1999).

After the degradation of the polyubiquitylated protein the proteasome releases small peptides derived from the substrate as well as reusable ubiquitin (Figure 5, right pannel).

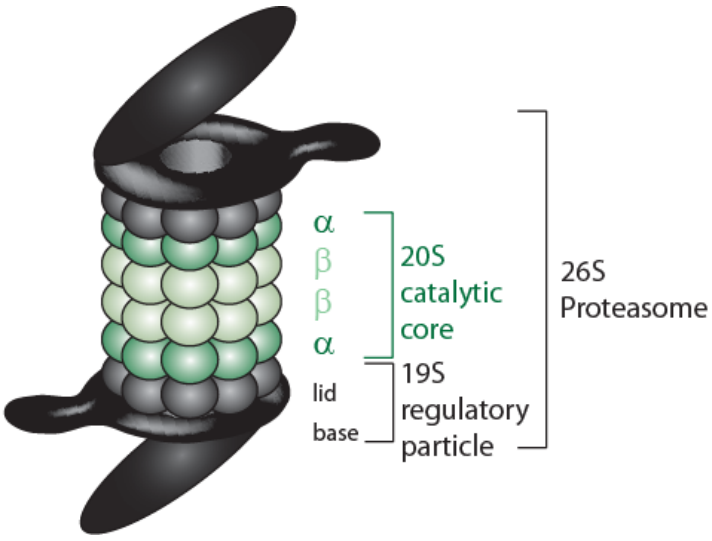

Figure 7| Schematic representation of the 26S proteasome.
The 26S proteasome comprises of (i) the barrel-like 20S catalytic core which is built by two β-rings and two α-rings and (ii) the two 19S particles, each consisting of the base and the lid, that cap either side of the barrel

1.7.3. Deubiquitylation of the substrate

Polyubiquitylation of proteins is a reversible process. Deubiquitylation is mediated by specialized proteases that are called deubiquitylating enzymes (DUBs). DUBs belong to a large and diverse group of proteases that specifically cleave ubiquitin from ubiquitin-conjugated protein substrates, ubiquitin precursors, ubiquitin adducts and polyubiquitin (reviewed in (Ventii and Wilkinson 2008). For deubiquitylation of a substrate, the DUB either hydrolyzes the amide bond between the terminal ubiquitin and the substrate or cleaves the isopeptide bond formed between two ubiquitin molecules (Pickart and Rose 1985; Wilkinson 1997; D'Andrea and Pellman 1998). Based on their molecular size, sequence homology, and active site residues, DUBs are categorized as UCHs (ubiquitin COOH-terminal hydrolyses) or UBPs (ubiquitin-specific proteases). In *S. cerevisiae*, there are at least 17 different DUBs, 16 of which are of the UBP family. None of those is encoded by an essential gene, suggesting that they have overlapping functions (Hochstrasser 1996).

Deubiquitylation has been implicated in numerous cellular functions, including cell cycle regulation (Song and Rape 2008), proteasome-and lysosome-dependent protein degradation (Guterman and Glickman 2004; Komada 2008), gene expression (Daniel and Grant 2007), DNA repair (Kennedy and D'Andrea 2005) and kinase activation (Adhikari, Xu et al. 2007; Komada 2008). Additionally, the DUBs are involved in maintaining the steady state levels of free ubiquitin and in affecting the stability of ubiquitylated proteins (Chung and Baek 1999; Wilkinson 2000). Finally, in *S. cerevisiae* two deubiquitylases, Rpn11 (POH1 in mammals) and Ubp6 (USP14 in mammals), have been shown to associate with the proteasome. These DUBs remove polyubiquitin chains from substrates during proteasomal degradation and upon inhibition of their function a monoubiquitylated substrate can transform into an efficient proteasome substrate (Guterman and Glickman 2004).

Although some substrates have been identified for a handful of DUBs, the substrates and physiological roles of most DUBs are poorly defined.

1.7.4. Non-proteolytic roles of the UPP in transcription

The proteasome is known to function in transcription independently of its proteolytic function. Initially, it was shown that the proteasomal ATPases of the 19S particle stimulate transcriptional initiation and elongation without engaging in protein degradation. Specifically, two 19S components, Sug1 and Sug2, were recruited to promoters but also to the entire ORF after gene activation, while components of the 20S particle were not (Ferdous, Gonzalez et al. 2001; Ferdous, Kodadek et al. 2002; Gonzalez, Delahodde et al. 2002; Sulahian, Sikder et al. 2006). Moreover, mutations in 19S components give rise to phenotypes consistent with an elongation defect (Ferdous, Gonzalez et al. 2001). Additionally, a genome-wide analysis of proteasome gene recruitment revealed that several hundred yeast genes had either the 20S or the 19S complex present but not both, suggesting some degree of independent function for the proteasomal sub-complexes (Sikder, Johnston et al. 2006). This finding was further corroborated by another genome-wide study showing the existence of a 19S-independent 20S complex and a 20S-independent ATPase-containing complex (Auld, Brown et al. 2006).

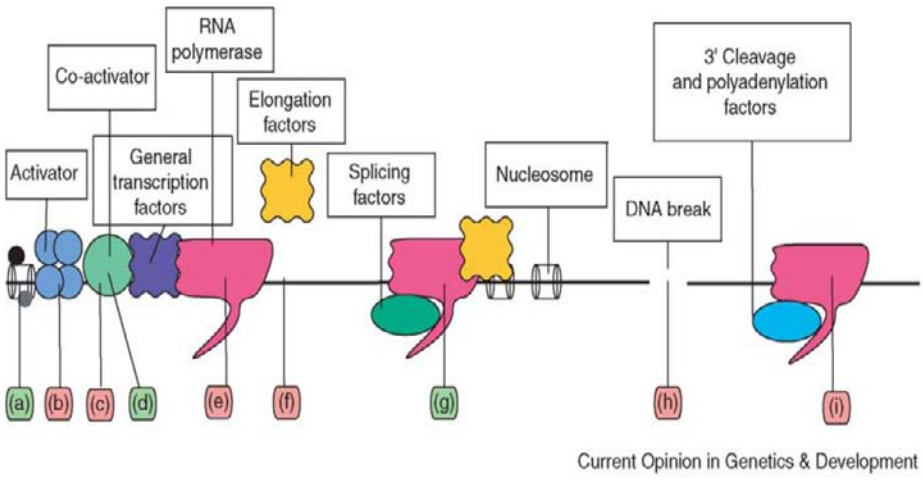

Figure 8| How the proteasome regulates transcription.

The proteasome regulates many aspects of transcription through both proteolytic (red) and non-proteolytic mechanisms (green). The non proteolytic roles include (a) interaction between the proteasome and chromatin (d) non-proteolytic stimulation of a co-activator and (g) promoting of efficient transcription elongation by 19S base subunits. Figure taken from (Collins and Tansey 2006).

Furthermore, since components of 19S genetically interact with the FACT elongation complex, which remodels histones during elongation, it was speculated that the unfolding activity of the 19S complex could facilitate elongation by promoting histone ejection, movement or exchange (Collins and Tansey 2006). In another study it was shown that the 19S complex can use the energy of ATP hydrolysis to drive stable complex formation of the SAGA complex during transcription initiation, indicating that the 19S can act both in early and late steps in the transcription process (Lee, Ezhkova et al. 2005). Other aspects of transcription are also influenced by 19S proteins since H3 methylation, a modification present in actively transcribed genes, requires proper function of the 19S components Rpt4 and Rpt6 (Ezhkova and Tansey 2004). Another non-proteolytic function of the UPP in transcription is the stimulation of transcription activators by monoubiquitylation (Salghetti, Caudy et al. 2001; Greer, Zika et al. 2003; Rajendra, Malegaonkar et al. 2004). However, there are data showing that this mono-ubiquitylation may be coupled to polyubiquitylation events that result in their subsequent degradation by the proteasome ((Kodadek, Sikder et al. 2006) and references therein). This is also supported by observations where activators and other transcription factors must be recycled regularly in a proteasome-dependent fashion to achieve high level of gene expression (Lee, Ezhkova et al. 2005; Lipford, Smith et al. 2005).
Taken together, it has become evident that non-proteolytic, as well as proteolytic, roles of the UPP are necessary for the transcription of many genes.

1.8. UBIQUITYLATION AND PROTEASOME-MEDIATED DEGRADATION OF RNAPII UPON DNA DAMAGE

The integrity of the genetic information, *i.e.* of cellular DNA, is constantly threatened by both endogenous and exogenous sources, including oxygen radicals within cells, environmental UV light, ionizing radiation and other genotoxic agents. All these sources can lead to DNA damage (Ljungman and Lane 2004). In turn, DNA damage can result not only in the inheritance of compromising genetic mutations, but it can also lead to significant and immediate changes of gene expression (Putnam, Jaehnig et al. 2009).

More importantly, bulky DNA adducts that occur within transcribed regions of the genome can cause the irreversible arrest of RNAPII, which blocks the expression of the damaged ORFs and consequently promotes cell death (Figure 9C) (Svejstrup 2003).

More than 20 years ago it was recognized that DNA lesions in the transcribed strand of an active gene are removed at a much higher rate than those in the non-transcribed strand or in the genome overall (Bohr, Smith et al. 1985; Mellon, Spivak et al. 1987). The cellular mechanism responsible for this removal is called Transcription Coupled Repair (TCR). TCR –a highly conserved process from *E. coli* to human– is a type of nucleotide excision repair (NER) that uses transcribing RNAPII as a sensor of DNA damage and directs the NER machinery to damaged sites in the genome ((Hanawalt and Spivak 2008) and references therein). This process is most likely triggered by the arrest of RNAPII at the site of DNA damage (Svejstrup 2007) and it allows rapid removal of DNA adducts from active genes (Figure 9A). Thus, TCR is an important cellular response for ensuring continued transcription. In humans, TCR is mediated by Cockayne syndrome A and B proteins (CSA and CSB) (Bregman, Halaban et al. 1996), while in *S. cerevisiae* by Rad26, the homolog of human CSB (van Gool, Verhage et al. 1994), and by Rpb9, a nonessential subunit of RNAPII (Li and Smerdon 2002).

However, if TCR fails the stalled RNAPII is removed from the site allowing the more general Global Genome Repair (GGR) pathway to repair the DNA damage (Figure 9B). The removal of RNAPII from the site of DNA damage is mediated by the UPP (Somesh, Reid et al. 2005). Polyubiquitylation and degradation of RNAPII was first thought to occur specifically in response to DNA damage (Bregman, Halaban et al. 1996; Ratner, Balasubramanian et al. 1998), but in fact this degradation of RNAPII is a ‘‘last resort” mechanism, used in order to clear active genes from persistently arrested RNAPII elongation complexes (Woudstra, Gilbert et al. 2002; Somesh, Reid et al. 2005). Specifically, in *S. cerevisiae* the switch from repair to degradation is mediated by the TCR protein Rad26 and the Degradation factor protein Def1 (Figure 9). It is believed that Rad26 inhibits RNAPII ubiquitylation or degradation, enabling the transcription block to first be sampled by TCR factors. If repair of the DNA damage fails Def1 promotes ubiquitylation or degradation of arrested polymerase complexes (Woudstra, Gilbert et al. 2002), so that repair can take place by GGR. Interestingly, in *S.cerevisiae* the positive

effect of Rad26 on RNAPII stability upon DNA damage is in contrast to the one observed in higher eukaryotes, where CSB (and CSA) are necessary for efficient RNAPII degradation upon DNA damage (Bregman, Halaban et al. 1996; Ratner, Balasubramanian et al. 1998).

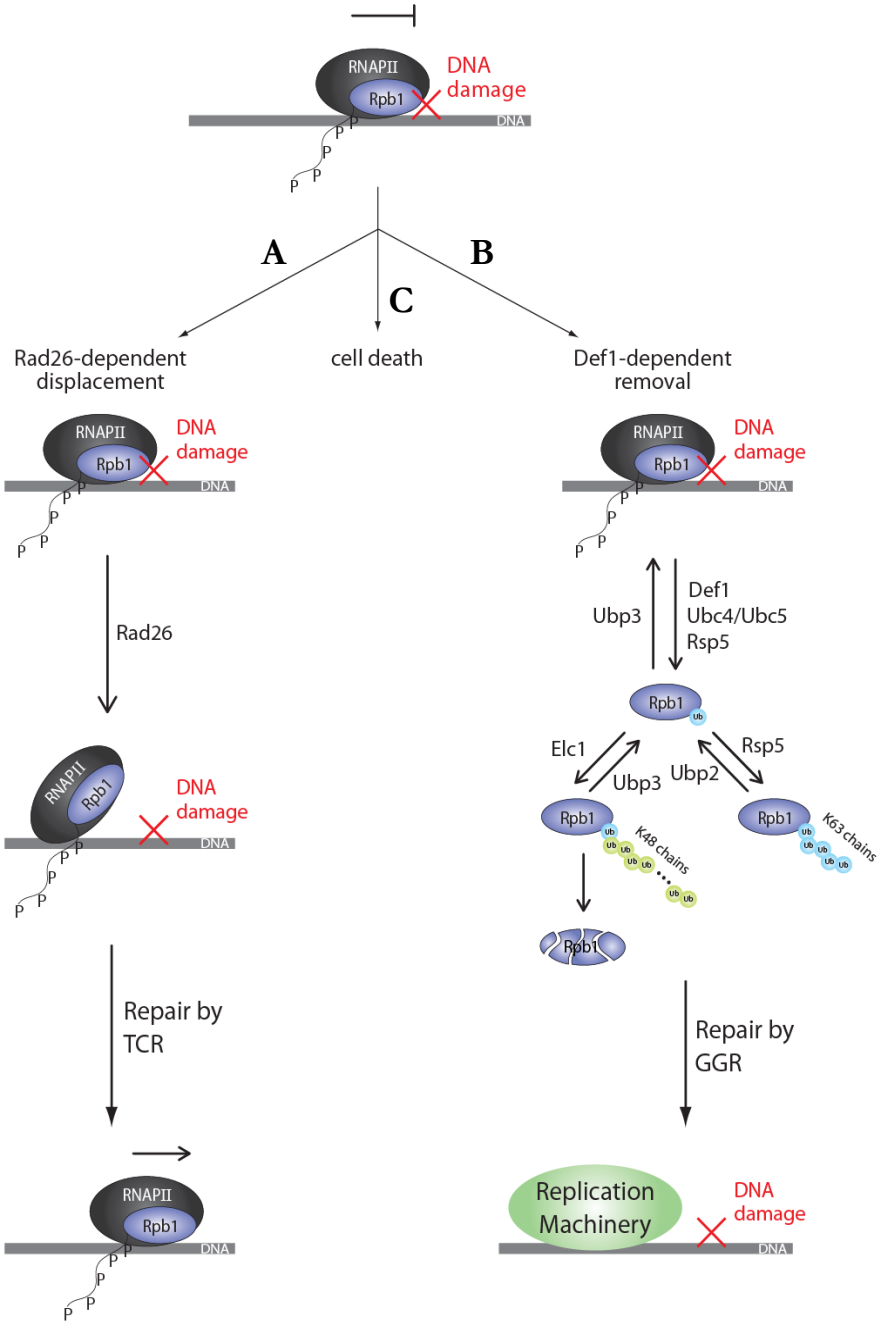

Figure 9 | DNA damage induces either TCR or UPP-mediated removal of RNAPII.
Upon DNA damage the transcribing complex becomes irreversibly stalled. Blocking of transcription might lead to cell death (**C**). To ensure continuation of transcription, the damage is either repaired by TCR (**A**) or as a "last resort" mechanism RNAPII is polyubiquitylated and degraded by the UPP, an action which allows the GGR pathway to repair the damage (**B**). See text for details.

The molecular mechanism for the polyubiquitylation of RNAPII upon DNA damage is well characterized by a number of studies in several different experimental systems (summarized in Figure 9B) (Bregman, Halaban et al. 1996; Beaudenon, Huacani et al. 1999; Gillette, Gonzalez et al. 2004; Somesh, Reid et al. 2005; Somesh, Sigurdsson et al. 2007; Kvint, Uhler et al. 2008; Yasukawa, Kamura et al. 2008; Harreman, Taschner et al. 2009). For degradation of RNAPII, its largest subunit Rpb1 is polyubiquitylated by the ubiquitin-conjugating enzymes (E2s) Ubc4 and Ubc5 and the ubiquitin ligases (E3s) Rsp5 and Elc1 (Beaudenon, Huacani et al. 1999; Reid and Svejstrup 2004; Somesh, Reid et al. 2005; Ribar, Prakash et al. 2007; Malik, Bagla et al. 2008; Harreman, Taschner et al. 2009). Unlike many E2s, Ubc5 plays an active role in the recognition of its substrate and binds directly to Rpb1 via a region called the "switch 2" domain of RNAPII (Somesh, Sigurdsson et al. 2007). Critically, this domain only becomes structured when the polymerase is in its elongating form (Cramer, Bushnell et al. 2001). By recognizing a structural feature within RNAPII that is determined by its activity, Ubc5 can direct its ubiquitylation abilities preferentially to the DNA/RNAPII/RNA ternary complex. Along these lines, the HECT E3 ligase, Rsp5, can only bind RNAPII via the Ser2 phosphorylated form of the CTD (Huibregtse, Yang et al. 1997; Somesh, Reid et al. 2005). Thus, Rsp5 uses the phosphorylation state of the CTD of Rpb1 to sense the transcriptional status of RNAPII. The combined action of Ubc5 and Rsp5 in directing ubiquitylation of only elongating RNAPII ensures that DNA damage will not cause unnecessary degradation of cellular polymerase, but rather only of polymerase that needs to be cleared away to restore normal transcription.

The E2s Ubc4 and Ubc5 function redundantly, whereas the E3s Rsp5 and Elc1 function sequentially, with Rsp5 mono-ubiquitylating Rpb1 and Elc1 adding a K48-linked polyubiquitin chain. In addition, Rsp5 produces non-functional K63-linked polyubiquitin chains (Harreman, Taschner et al. 2009). The polyubiquitin chain is attached in two distinct ubiquitylation sites on Rpb1, K330 and K695. These two sites are more than 125 Å apart and their modification is coordinated *in vitro* as well as *in vivo*, suggesting that an E2/E3/RNAPII complex is assembled that depends on not only the RNAPII CTD but also on the Rpb1 ubiquitylation sites themselves (Somesh, Sigurdsson et al. 2007).

The polyubiquitylated Rpb1 is then degraded by the 26S proteasome. Rpb1 is the only RNAPII subunit degraded upon DNA damage (Malik, Bagla et al. 2008). Through the specific degradation of Rpb1, the stalled complex is most likely released from the site of the DNA damage and the damage becomes accessible for repair. However, when the DNA damage is repaired before Rpb1 is degraded, polyubiquitylated Rpb1 is deubiquitylated by the ubiquitin proteases Ubp2 and Ubp3 and spared from degradation (Kvint, Uhler et al. 2008; Harreman, Taschner et al. 2009). Rsp5 and Ubp2 interact with each other (Kee, Lyon et al. 2005; Lam, Urban-Grimal et al. 2009), and Ubp2 is capable of hydrolyzing K63 chains to produce mono-ubiquitylated RNAPII. In contrast to Ubp2, Ubp3 'rescues' RNAPII from degradation by hydrolyzing K48-linked ubiquitin chains (Kvint, Uhler et al. 2008) and by producing completely de-ubiquitylated polymerase. Thus, Ubp2 and Ubp3 appear to have temporally distinct roles in the deubiquitylation of RNAPII upon DNA damage.

Taken together, upon DNA damage there is an immediate cellular response to restore the genetic information via TCR. However, if this fails the cell employs the "last resort" mechanism of polyubiquitylation and degradation of the stalled RNAPII. This action enables the more general GGR pathway to repair the damage thus allowing efficient transcription of the gene (Figure 9B).

1.9. AIM OF THIS STUDY

Continued transcription is important for the survival of the cell. Transcription is one of the most regulated and complex cellular processes. Regulation occurs via the function of a large number of proteins (Arndt and Kane 2003; Saunders, Core et al. 2006; Hirose and Ohkuma 2007; Panning and Taatjes 2008; Fuda, Ardehali et al. 2009). To that end it is not surprising that the causes of several human diseases have been attributed to transcriptional impairment (Conaway and Conaway 1999; Engelkamp 2000). Transcription elongation is a highly complex and discontinuous process including frequent pausing of the mRNA synthesizing enzyme RNAPII (Neuman, Abbondanzieri et al. 2003; Darzacq, Shav-Tal et al. 2007; Ardehali and Lis 2009; Pelechano, Jimeno-Gonzalez et al. 2009). Interestingly, it became evident that RNAPII pausing and arrest is a common phenomenon observed *in vivo* in about 4% of the polymerases that enter elongation(Darzacq, Shav-Tal et al. 2007). In order to reduce the negative effects of such events and to prevent irreversible arrest of the transcribing complex, a number of proteins have been reported to allow RNAPII to resume transcription (Awrey, Weilbaecher et al. 1997; Nesser, Peterson et al. 2006; Ardehali, Yao et al. 2009).

But what if this fails? In this case the irreversibly stalled RNAPII would block transcription of the gene and compromise cell viability. So, the arrested RNAPII has to be recognized and degraded to free the gene for subsequent polymerases. The cellular pathway for removal of these RNAPII complexes is not known.

The aim of this study was to elucidate the molecular mechanism employed by the cell upon transcriptional stalling of RNAPII. Initially, it should be assessed whether the transcriptionally stalled RNAPII complex is recognized and degraded by the UPP. Furthermore, for UPP-mediated degradation to take place a polyubiquitin chain has to be attached to RNAPII. To that end, this study aimed to identify which ubiquitin-modifying enzymes are involved in the poly- and de-ubiquitylation of RNAPII. Moreover, since UPP-mediated degradation of RNAPII is also a cellular response to DNA damage it should be addressed whether the two pathways employed by the cell are identical, overlapping or completely different. In fact, since Ubc4 and Ubc5 are needed for polyubiquitylation of Rpb1 in response to transcriptional stalling (Somesh, Reid et al.

2005), it was speculated that transcriptionally stalled RNAPII complexes are degraded by the same pathway as RNAPII stalled due to DNA damage (Somesh, Reid et al. 2005; Daulny and Tansey 2009).

More importantly, it is not known whether transcription elongation impairment in general, which has been proposed to increase the events of natural pausing, results in the UPP-mediated degradation of RNAPII. To that end, this study aimed to assess whether temperature stress as well as depletion of the nucleotide pool –two phenomena frequently observed in nature– result in RNAPII degradation similarly to the lack of functional auxiliary transcription elongation factors.

Finally, since there is a functional and structural resemblance of the three major RNA polymerases –RNAPI, RNAPII and RNAPIII– (Archambault and Friesen 1993) this study aimed to address whether the specific degradation of the transcribing complex upon transcriptional stalling is also present in the other two major transcribing complexes, RNAPI and RNAPIII.

Taken together, the aim of this study was to discover and elucidate the molecular mechanism used by the cell for removal of transcriptionally stalled RNAP complexes.

2. RESULTS

Transcription elongation is a highly dynamic and discontinuous process that includes frequent pausing of RNAPII. Paused RNAPII complexes resume transcription with the help of transcription elongation factors. If transcription elongation factors fail to restart RNAPII, the persistently stalled RNAPII complex prevents transcription and has to be recognized and degraded by the cell. The cellular pathway for removal of these RNAPII complexes is not known.

My goal was to study the transcriptional stalling-dependent degradation of RNAPII. To elucidate the molecular mechanism for this degradation, it was hypothesized that stalling of RNAPII and consequently degradation of Rpb1 is enhanced when transcription elongation is impaired. In order to impair transcription elongation 4 non-essential transcription elongation factors were deleted: Ctk1, Bur2, Tho2 and Dst1. Ctk1 is the kinase subunit of the CTDK-I complex that phosphorylates the CTD of Rpb1, Dst1 is the cleavage factor TFIIS, Tho2 is a subunit of the THO complex, and Bur2 is the cyclin of the Bur1-Bur2 kinase complex. Deletions of all factors are viable but, except of *Δdst1*, the mutants display an impaired growth phenotype under normal conditions (Figure 10).

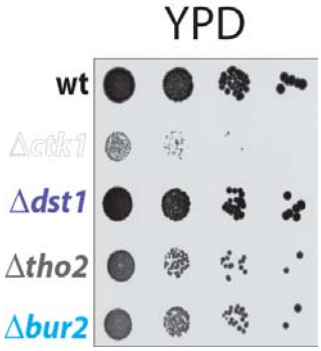

Figure 10 | Deletion of transcription elongation factors leads, except of *Δdst1*, to an impaired growth phenotype.
10-fold serial dilutions of wild-type (wt), Δ*ctk1*, Δ*dst1*, Δ*tho2* and Δ*bur2* cells were spotted onto YPD plates and incubated at 30°C for 3 days.

2.1 IMPAIRMENT OF TRANSCRIPTION ELONGATION RESULTS IN THE DEGRADATION OF RPB1, THE LARGEST SUBUNIT OF RNAPII

2.1.1 Transcription elongation impairment results in decreased RNAPII occupancy on the gene.

To assess the recruitment of RNAPII to genes in the transcription elongation mutants Chromatin ImmunoPrecipitation (ChIP) assays were performed using *RPB3-TAP* tagged strains. The recruitment was tested in three exemplary highly transcribed genes, *ADH1*, *ACT1*, and *PMA1*. In the case of *ADH1* a set of 4 primers was used that span from the promoter to the 3' region of the ORF, allowing the detailed analysis of RNAPII's recruitment (Figure 11A). RNAPII occupancy decreases about 2-fold in the transcription elongation mutants on all three genes compared to the one observed in wild-type cells (Figure 11B). The observed reduction in the levels of RNAPII present on the gene is probably resulting from the fact that the proteins deleted in these mutants have been shown to be required for promoting efficient RNAPII transcription (see Introduction 1.6).

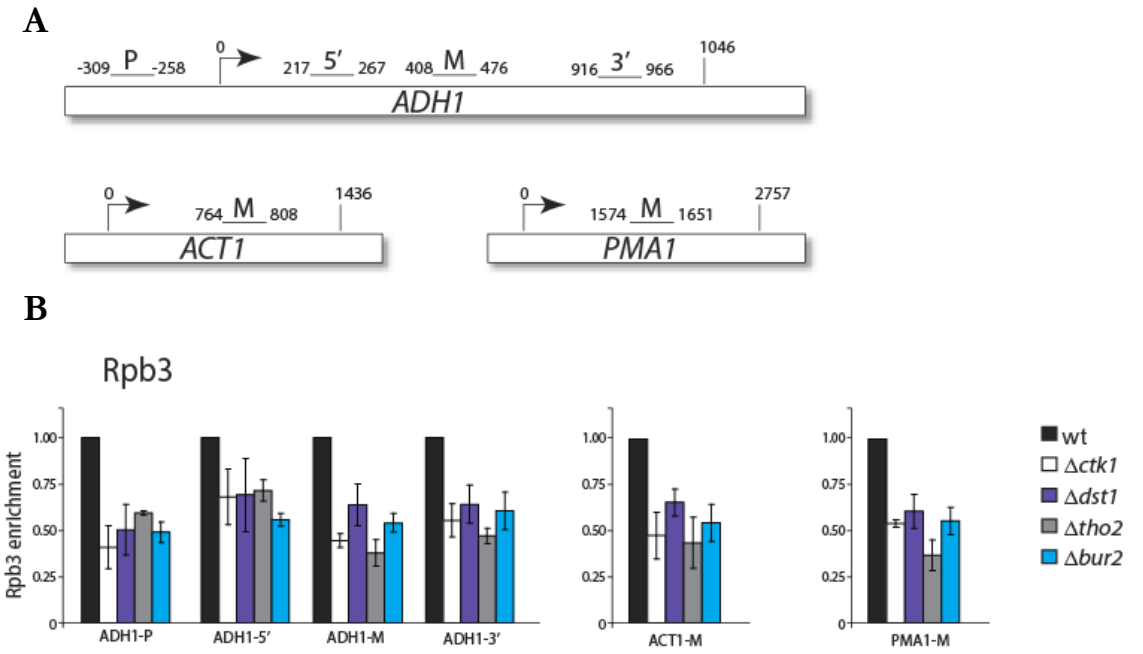

Figure 11 | RNAPII occupancy on the gene is decreased in Δ*ctk1*, Δ*dst1*, Δ*tho2* and Δ*bur2* cells.
ChIP experiments were performed with an *RPB3-TAP* strain. **A,** The indicated primers were used to assess recruitment to the genes *ADH1*, *ACT1*, and *PMA1*. **B,** Enrichment of Rpb3 on these genes was quantified by RealTime-PCR with the primers shown in A. As a control, primers for a non-transcribed region (NTR) of chromosome V were used. PCR efficiencies (E) were determined with standard curves. Enrichment of Rpb3 over the NTR was calculated according to $[E^{(C_T \text{ Input} - C_T \text{ IP})}]_{gene}$ / $[E^{(C_T \text{ Input} - C_T \text{ IP})}]_{NTR}$. Columns and error bars represent the mean ± standard deviation from 3 independent experiments.

2.1.2 Transcription elongation impairment results in lower Rpb1 protein levels.

Reduction in the occupancy of a protein on the gene might result from its inefficient recruitment to the site of transcription. To investigate whether the recruitment of RNAPII was responsible for its reduced presence on the gene, the protein levels of Rpb1 were assessed in the four transcription elongation mutants. Interestingly, deletion of any of these four transcription elongation factors results in reduced total cellular Rpb1 levels to about 50% (Figure 12). Rpb1 levels were detected by quantitative western blot (qWB) analysis and normalised against the levels of the protein Pgk1 that served as a loading control. The observed reduction was independent of the specificity of the antibody used for detection of Rpb1 since the same extent of reduction was observed either with the 8WG16 antibody or the yN-18 antibody recognizing the C- or the N-terminus, respectively (Figure 12A and B). This indicates that the total cellular levels of Rpb1 are reduced upon deletion of these transcription elongation factors.

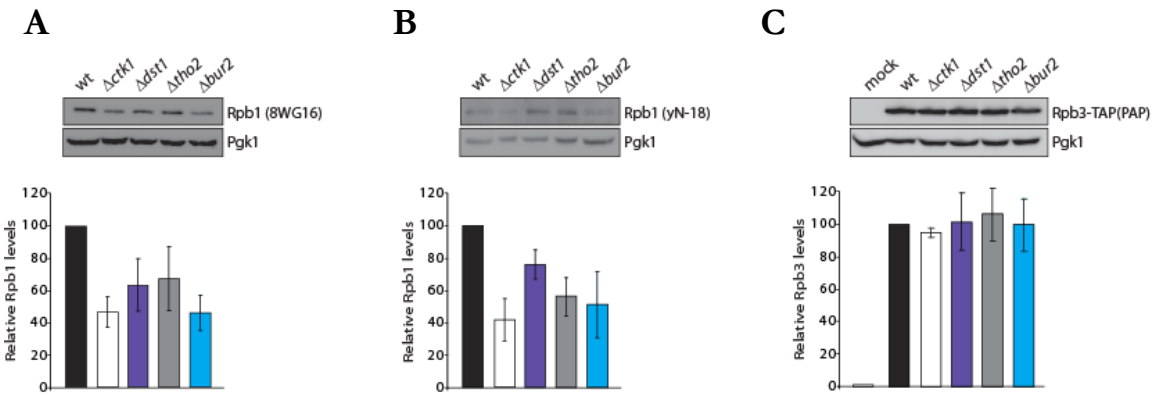

Figure 12 | Total cellular levels of Rpb1 are specifically reduced.

For quantification of the total cellular levels of RNAPII subunits, whole cell extracts of the indicated yeast strains were subjected to quantitative western blot analysis (qWB). Western blot signals were acquired using the Fujifilm Mini-LAS300 System (Fujifilm Life Sciences) and quantified using the MultiGauge ScienceLab2005Ver3 (Fujifilm Life Sciences). **A,** and **B,** Total cellular levels of Rpb1 are reduced in Δ*ctk1,* Δ*dst1,* Δ*tho2* and Δ*bur2* cells as assessed by probing with the 8WG16 and yN-18 antibodies. **C,** Protein levels of Rpb3-TAP remain unaffected as assessed by probing with PAP. Levels of total Rpb1 and Rpb3 were normalized to Pgk1. Columns and error bars represent the mean ± standard deviation from 4 independent experiments (bottom panels).

To investigate whether the levels of Rpb3 are also affected upon transcription elongation impairment the total cellular levels of Rpb3 were quantified in wild-type and the four transcription elongation mutants. There was no observed reduction in the Rpb3 protein levels upon deletion of the four transcription elongation factors (Figure 12C). This indicates that RNAPII's biggest subunit, Rpb1, is specifically degraded and through this degradation probably the entire enzyme is then disassembled. Consistently, in response to DNA damage there is specific degradation of Rpb1 but not of other subunits of elongating RNAPII (Malik, Bagla et al. 2008).

Furthermore, deletion of a transcription elongation factor could affect the transcription of either *RPB1* and/or *RPB3* and thus indirectly lead to reduced Rpb1 levels. In order to exclude this, the mRNA levels of *RPB1* and *RPB3* were quantified and normalised to the mRNA levels of 2 housekeeping genes *ADH1* and *ACT1*. To that end, total RNA was extracted from wt and *Δctk1* cells –the mutant with the slowest growth rate– and after reverse transcription, the levels of each mRNA were quantified by Real-Time PCR. There was no specific reduction of either the *RPB1* or the *RPB3* mRNA in the *Δctk1* cells (Figure 13). This shows that the decrease in the protein levels of Rpb1 is not caused by reduced levels of *RPB1* mRNA.

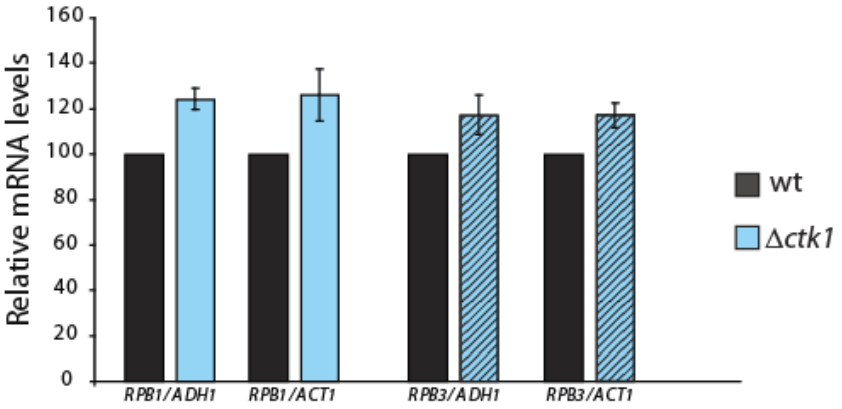

Figure 13 | Levels of mRNAs encoding *RPB1* and *RPB3* are not reduced compared to mRNAs encoding the house-keeping proteins Adh1 and Act1 in *Δctk1* cells

Total mRNA levels encoding *RPB1, RPB3, ACT1* and *ADH1* mRNA were quantified by Real-Time PCR in wild-type and *Δctk1* cells. Relative *RPB1* and *RPB3* mRNA levels were calculated using a standard curve method as the ratio of the *RPB1* and *RPB3* over the *ADH1* or *ACT1* mRNA amount. Columns and error bars represent the mean ± standard deviation from 3 independent experiments

2.1.3 Transcription elongation impairment does not result in the reduction of the protein levels of other transcription factors.

During transcription a number of transcription factors are bound to the transcribing RNAPII complex. From the above experiments it became evident that upon transcriptional stalling there is specific degradation of Rpb1. To investigate whether there is also UPP-mediated degradation of associated factors, the protein levels of Spt5, Med2 and Dst1 were quantified upon transcriptional stalling.

Spt5 is part of the Spt4/Spt5 complex required for efficient transcription elongation and is bound to RNAPII throughout transcription elongation (Hartzog, Wada et al. 1998; Lindstrom and Hartzog 2001). Med2 is part of the Mediator complex which associates with RNAPII and is important for the transduction of activation signals from enhancer bound activators to general transcription factors (Guglielmi, Soutourina et al. 2007; Esnault, Ghavi-Helm et al. 2008; Fan and Struhl 2009). TFIIS is the general elongation factor that stimulates the intrinsic cleavage activity of RNAPII which then enables the arrested complex to backtrack (Awrey, Shimasaki et al. 1998; Kettenberger, Armache et al. 2003; Wang, Bushnell et al. 2009).

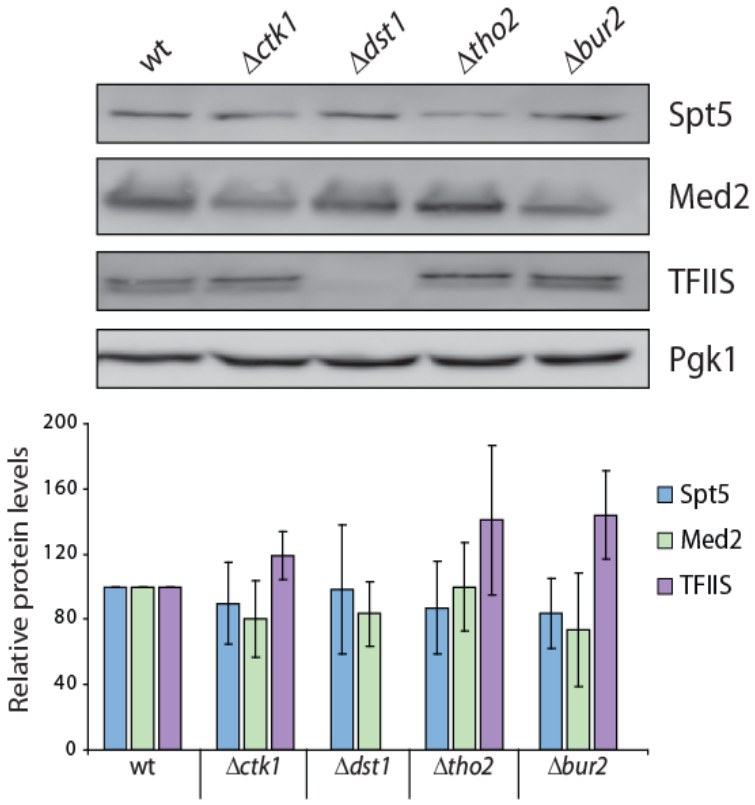

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Figure 14 | Protein levels of Spt5, Med2 and Dst1 remain are not reduced upon transcriptional stalling. Total cellular levels of Spt5, Med2 and Dst1 are not reduced upon deletion of Ctk1, Dst1, Tho2, and Bur2. Experiment as in Figure 12. Columns and error bars represent the mean ± standard deviation of 3 independent experiments (bottom panels).

As shown in Figure 14, there was no significant reduction in the protein levels of any factor observed. This indicates that the associated transcription factors might also disassemble from the arrested complex after degradation of Rpb1 –similarly with the other polymerase subunits.

2.1.4 Depletion of transcription elongation factors also leads to lower Rpb1 levels.

As deletion of the transcription elongation factors Ctk1, Tho2 and Bur2 leads to an impaired growth phenotype (Figure 10), the observed decrease in the Rpb1 protein levels could be a secondary effect, caused by an accumulation of various defects. To exclude this possibility, a genomic depletion system for these genes was used based on a strain carrying a C-terminal TAP-tagged version of each protein (to be able to determine protein levels using the PAP antibody) driven by the *GAL1* promoter (*GAL1::GENE-TAP*). In galactose-containing media (YPG) the protein will be expressed, whereas in glucose-containing media (YPD) its expression will be repressed (Figure 15A).

The growth rates between the wild-type and the *GAL1::GENE-TAP* strains were identical for the first 8 hours after shift from YPG to YPD, whereas after 8 hours some of the depleted strains started to deviate from wt growth (Figure 15B). More importantly after the 8 hour depletion in YPD media none of the 3 proteins were detectable by western blotting (Figure 15C). Therefore, the 8 hour depletion time point was selected for assessing the total cellular Rpb1 levels. As shown in Figure 15D, depletion of Ctk1, Tho2, or Bur2 resulted into the same reduction of total Rpb1 levels at a time point when (i) growth of the cells was not impaired and (ii) the proteins were not present in the cell. This

indicates that the observed reduction in the Rpb1 protein levels is a direct effect of transcription elongation impairment.

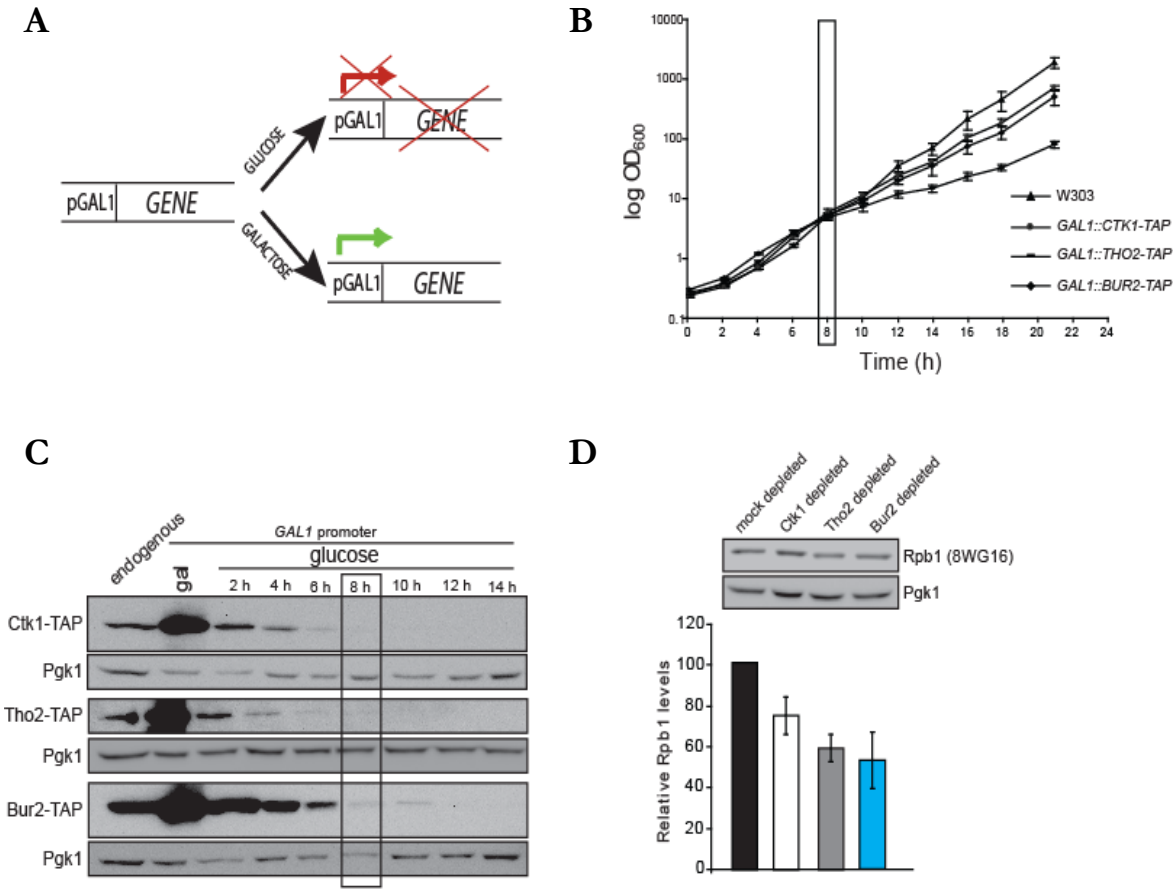

Figure 15 | Depletion of transcription elongation factors results in lower total levels of Rpb1.
A, Schematic representation of the GAL depletion system. **B,** Growth curve of yeast cells depleted of Ctk1, Tho2, or Bur2. Growth is not impaired during the first 8 hours of depletion. **C,** Protein levels of Ctk1, Tho2, and Bur2 after different depletion times. Depletion of TAP-tagged versions of these proteins was assessed by western blotting using PAP antibody. **D**, Total levels of Rpb1 were reduced after 8 hours of depletion of Ctk1, Tho2, or Bur2. Columns and error bars represent the mean ± standard deviation from 3 independent experiments.

2.1.5 Treatment with the transcription elongation inhibitor 6AU results in lower Rpb1 levels.

Treatment with 6AU, a drug that decreases cellular GTP/UTP levels, also leads to transcription elongation impairment of RNAPII (Exinger and Lacroute 1992). Treatment

of the yeast cells with 6AU hence allows a more short-term inhibition of transcription elongation and probably mimics natural conditions of starvation. To assess whether this inhibition results in decreased Rpb1 levels as observed upon deletion or depletion of the transcription elongation factors the cellular levels of Rpb1 were quantified before and after treatment with 6AU.

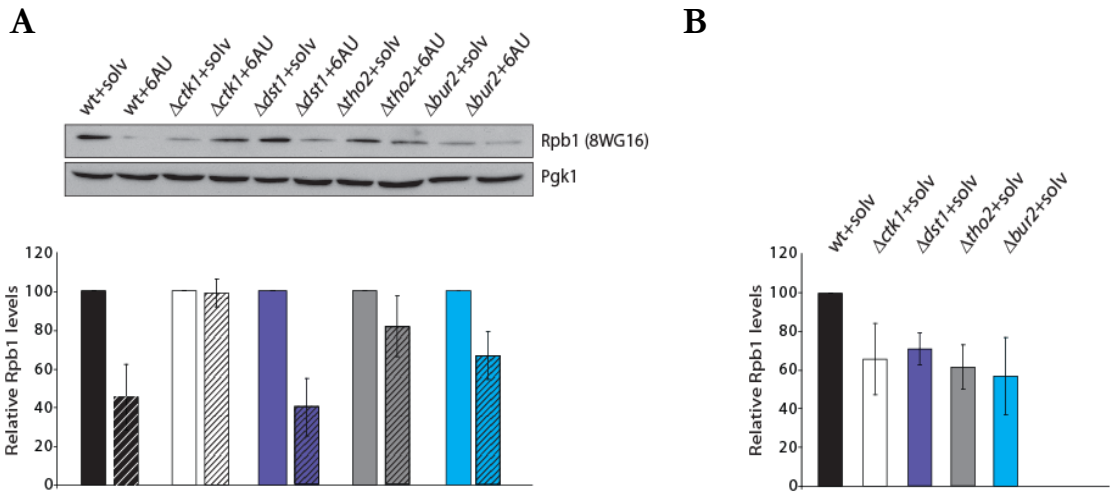

Figure 16 | Treatment with 6AU results in lower total levels of Rpb1.
The indicated yeast cells were treated (+6AU) or not (+solv) with the transcription elongation drug 6AU. Experiment as in Figure 12. **A,** 6AU leads to decreased total cellular levels of Rpb1 in wild-type and *Δdst1* and *Δbur2* cells. **B,** Quantification of Rpb1 levels in cells treated with just the solvent of 6AU shows a similar reduction in the protein levels as observed in Figure 12. Columns and error bars represent the mean ± standard deviation from 3 independent experiments.

Two hours incubation with the drug in wild-type cells reduced total cellular Rpb1 levels to about 50% (Figure 16A, compare first 2 lanes). Moreover, 6AU treatment of the deletion mutants resulted in an additional reduction of the Rpb1 levels for the *Δdst1* and *Δbur2* cells but not for the other two mutants (Figure 16A). Such an observation indicates that probably there is a threshold of lower Rpb1 levels that is essential for viability, which was already reached in the *Δctk1* and *Δtho2* cells. This hypothesis would explain why addition of 6AU could not cause any further reduction in the Rpb1 levels in these strains.

Taken together, total cellular Rpb1 levels are specifically reduced upon transcription elongation impairment.

2.2 REQUIREMENT OF THE CTD OF RPB1 IN THE DEGRADATION OF TRANSCRIPTIONALLY STALLED RNAPII

During transcription initiation RNAPII frequently stops and stalls. So, the transcribing complex should be protected from UPP-mediated degradation during this phase. Several lines of evidence point to the CTD of Rpb1 as being important to protect the complex during initiation. Specifically, it was found that (i) RNAPII with a CTD phosphorylated on Ser5, a hallmark of initiation, is not polyubiquitylated *in vitro* (Somesh, Reid et al. 2005), (ii) the full-length CTD (26 repeats), but not a truncated 12-repeat CTD, allows the assembly of the ubiquitylation machinery for efficient Rpb1 polyubiquitylation, (iii) the inactivation of the Ser5-specific phosphatase Ssu72 significantly decreases damage-induced RNAPII degradation (Somesh, Reid et al. 2005), (iv) hyperphosphorylated Rpb1 is ubiquitylated *in vitro* in higher eukaryotes (Mitsui and Sharp 1999) and (v) α-amanitin causes degradation of hyperphosphorylated Rpb1 *in vivo* (Arima, Nitta et al. 2005).

To investigate the requirement of the CTD in degradation of transcriptionally stalled RNAPII, the CTD of Rpb1 was either truncated or mutated in the transcription elongation mutants. In particular, a set of four truncations (with the shortest being 10 heptapeptide repeats) and two mutations (affecting either Ser2 or Ser5 phosphorylation) of the CTD were used (West and Corden 1995). The viability of the transcription elongation mutants that also carried those CTD truncations or mutations varied (Table 2). As the CTD is responsible for the recruitment of different proteins and thus for the progression through the transcription cycle (see Introduction 1.3) the growth variations are probably reflecting the function of Ctk1, Dst1, Tho2 and Bur2 in different stages of transcription.

If proper CTD function is a prerequisite for efficient ubiquitylation and degradation of Rpb1 of transcriptionally stalled RNAPII one would expect that mutating or truncating the CTD would affect total cellular Rpb1 levels. To that end, the Rpb1 levels were quantified in the combined mutant strains by qWB (Figure 17).

Table 2 | Growth analysis of the combination of the transcription elongation mutants with CTD mutations or truncations.

Two types of CTD mutants (grey) and four types of CTD truncations (green) were introduced in the deletion mutants of Ctk1, Dst1, Tho2 and Bur2. For the CTD mutations, in the indicated number of repeats either the serine 5 or the serine 2 of the heptapeptide was mutated to alanine. Occasionally, the combination of both mutations resulted into a synthetic lethality phenotype (-). However, most double mutans were viable (+).

Transcription Factor / *CTD Variant*	*wt*	*Δctk1*	*Δdst1*	*Δtho2*	***Δbur2***
7wt 7A5 repeats	+	−	+	−	+
9wt 6A2 repeats	+	−	+	+	−
26 wt repeats	+	+	+	+	+
14 wt repeats	+	+	+	+	+
13 3/7 wt repeats	+	−	+	+	+
11 2/7 wt repeats	+	+	+	+	−
10 5/7 wt repeats	+	+	−	+	−

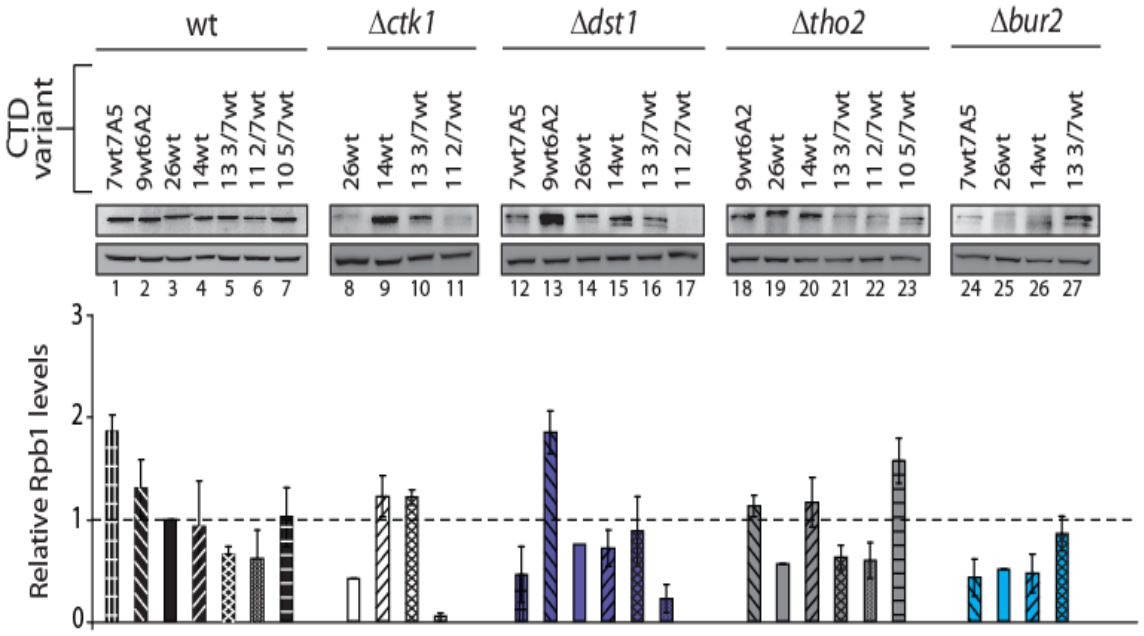

Figure 17 | Quantification of the total cellular Rpb1 levels in the transcription elongation mutants that also carry CTD mutations or truncations.

Total cellular Rpb1 levels were assessed in the indicating strains. Experiment as in Figure 12. A specific requirement of the CTD in the degradation of transcriptionally stalled Rpb1 is not evident. Columns and error bars represent the mean ± standard deviation from 3 independent experiments.

Specifically, in the mutant that affected the Ser2 phosphorylation (9wt6A2) there was restoration of the Rpb1 protein levels in the *Δdst1* and *Δtho2* cells (Figure 17, compare lanes 13 & 14; 18 & 19).

This could be explained by the fact that the elongating form of RNAPII (Ser2 phosphorylated on the CTD) is the preferred substrate for polyubiquitylation. Unfortunately, in the *Δctk1* and *Δbur2* cells this additional CTD mutation resulted in a synthetic lethal phenotype (Table 2) which might reflect the involvement of both the CTDK-I and the Bur1/2 complex in phosphorylating the CTD *in vivo*. Additionally, in the *Δctk1* cells that also carry the of the truncated CTD with either 14 or 13 and 3/7 wt repeats there were significantly higher Rpb1 levels (Figure 17, compare lanes 8-10). This could be a result of the loss of recruitment of transcription factors in these cells; a recruitment that could depend on the length of the CTD.

Taken together the above experiment provides hints that the state of the CTD of Rpb1 might be involved in the pathway for RNAPII ubiquitylation and degradation upon transcriptional stalling. However, further analysis is required for elucidating the molecular mechanism behind this involvement.

2.3 TEMPERATURE STRESS LEADS TO DEGRADATION OF RNAPII.

All organisms are exposed to changes in their environmental conditions. Increases or decreases of the ambient temperature are common and may take place seasonally, daily or just unexpectedly, depending on region, climate and environment. Several studies have investigated the response and adaptation of yeast cells to either a cold or a heat shock (Becerra, Lombardia et al. 2003; Aguilera, Randez-Gil et al. 2007; Auesukaree, Damnernsawad et al. 2009). In order to mimic potential transcription elongation impairment in more natural conditions the levels of Rpb1 were tested in yeast cells growing at temperatures from 18 °C to 42 °C for one hour. As shown in Figure 18, elevated growth temperatures –37°C and 42°C– resulted in decreased total cellular Rpb1 levels. The observed decrease was not as pronounced as upon deletion of the four

transcription elongation factors (Figure 12A) or upon nucleotide depletion (Figure 16A), but this probably reflects the ability of the cells to adapt to the elevated temperature.

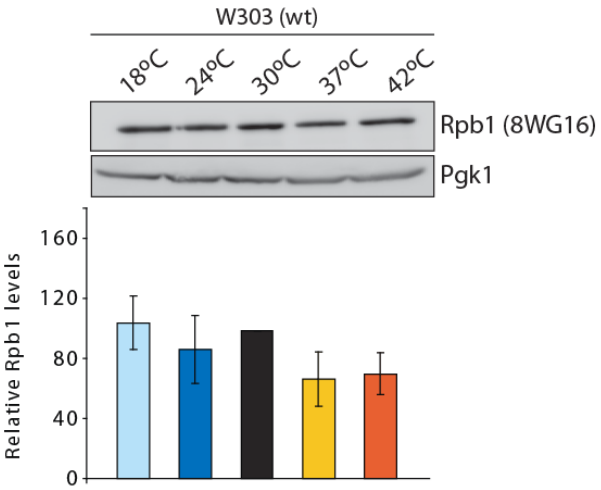

Figure 18 | Quantification of the total Rpb1 levels upon temperature stress.
Total cellular Rpb1 levels were assessed in wild-type cells grown at the indicated temperatures for one hour. Only growth in higher temperatures resulted in decreased cellular Rpb1 levels. Experiment as in Figure 12. Columns and error bars represent the mean ± standard deviation from 3 independent experiments.

The observed reduction in the Rpb1 levels might reflect the UPP-mediated degradation of the protein. To investigate whether proper proteasomal function is required for this reduction and how rapidly this is done, a time course experiment was performed. Specifically, the Rpb1 levels were quantified after 5, 10, 15 and 30 minutes after the temperature shift in the presence or absence of the proteasomal inhibitor MG132 (Figure 19).

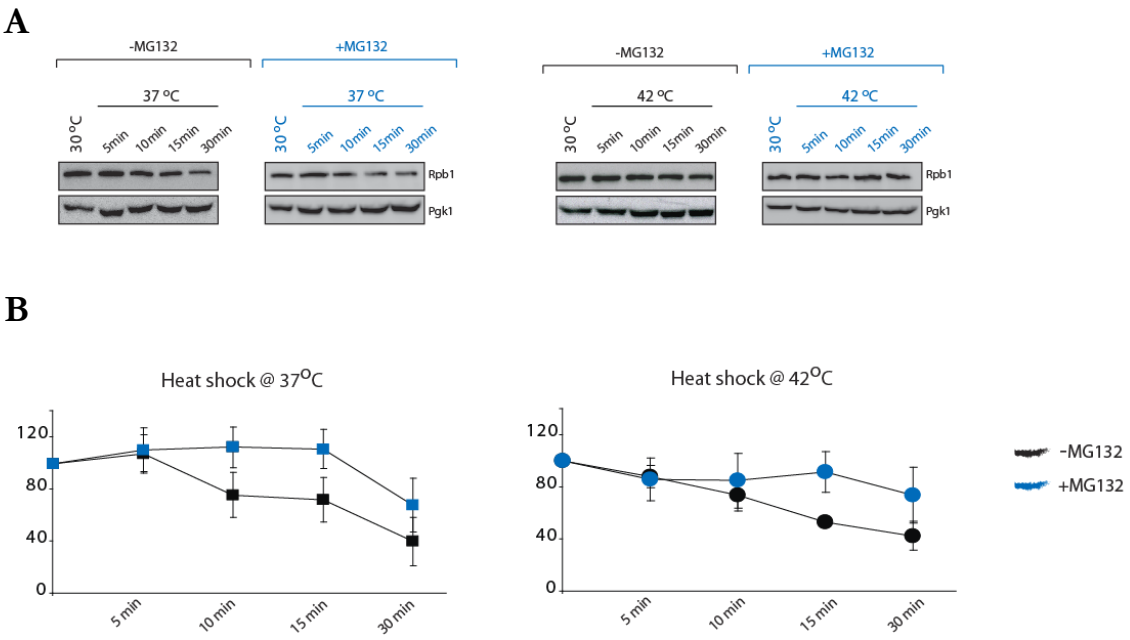

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Figure 19 | Quantification of the total cellular Rpb1 levels upon growth at 37°C and 42°C in the presence or absence of MG132.

Total cellular Rpb1 levels were assessed in wild-type cells grown at the indicated temperatures. For pre-treatment with the proteasomal inhibitor MG132, 100µM of the drug were added to the culture one hour prior to the temperature shift. **A,** Western blot signals of total protein extracts from wild-type cells grown as indicated. **B,** Quantification plots of **A**. Experiment as in Figure 12. Symbols (squares and circles) and error bars represent the mean ± standard deviation of 4 independent experiments.

Even after 10 minutes at 37°C and 5 minutes at 42°C there was a significant reduction in the levels of Rpb1 (Figure 19B, black symbols). More importantly, pre-treatment of the yeast culture with MG132, *i.e.* inhibition of proteasomal function prevented the reduction of Rpb1 levels observed after the temperature shift (Figure 19B, blue symbols). This suggests that indeed the proteasome is responsible for the degradation of Rpb1 after heat shock. However, after 30 minutes in both temperatures there is no difference observed in the Rpb1 levels of cells pre-treated or not with MG132.

Taken together higher growth temperatures, which might lead to "more natural" transcriptional impairment, results in the degradation of RNAPII's largest subunit. However, further analysis is required to assess whether the observed reduction is indeed specific UPP-mediated degradation after transcriptional stalling or is a result of the denaturation of the large Rpb1 protein after heat shock.

2.4 TRANSCRIPTIONALLY STALLED RNAPII IS POLYUBIQUITYLATED AND DEGRADED BY THE UBIQUITIN-PROTEASOME PATHWAY (UPP)

2.4.1 Transcriptionally stalled RNAPII is polyubiquitylated

The specific reduction in the total cellular Rpb1 levels suggests a targeted cellular degradation of the protein by the UPP (see Introduction 1.7). The first step of this pathway is the attachment of a polyubiquitin chain to the targeted substrate. To test

whether Rpb1 is polyubiquitylated upon transcription elongation impairment, RNAPII was purified from wild-type and the four transcription elongation mutants. The samples were then subjected to western blotting using anti-ubiquitin antibodies. This allowed quantification of the levels of polyubiquitylated Rpb1. Wild-type cells displayed a low level of polyubiquitylated Rpb1, which increased about two to four fold when transcription elongation was impaired (Figure 20).

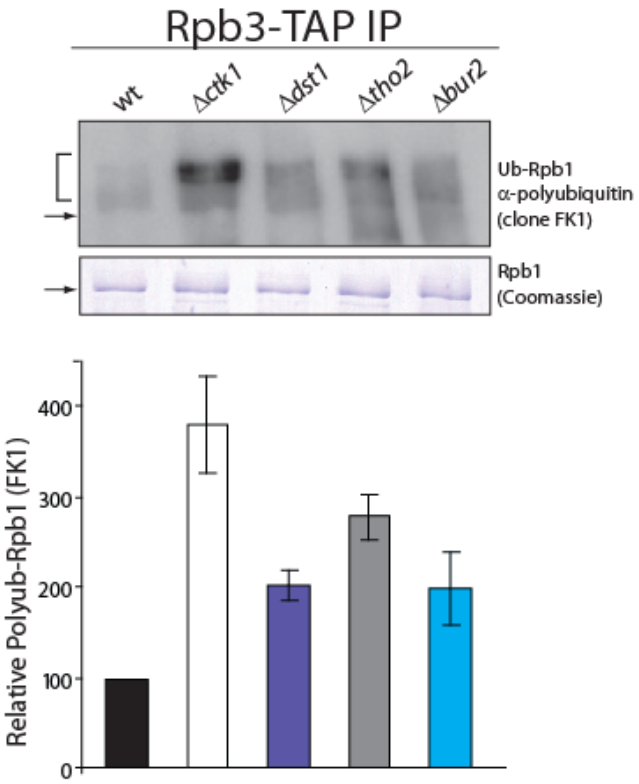

Figure 20 | Polyubiquitylation of Rpb1 is increased in *Δctk1*, *Δdst1*, *Δtho2*, and *Δbur2* cells.

RNAPII was purified using Rpb3-TAP. Polyubiquitylation of Rpb1 was assessed by western blotting using α-ubiquitin specific antibody (upper panel). The approximate positions of Rpb1 (arrow) and polyubiquitylated Rpb1 (brackets) are indicated. Columns and error bars represent the mean ± standard deviation from 3 independent experiments

Consistently, Rpb1 ubiquitylation is increased in *Δdst1* cells and after treatment with 6AU (Somesh, Reid et al. 2005). To further corroborate this finding, Rpb1 was purified under denaturing conditions that allowed purification of single RNAPII subunits (Figure 21A) from wild type cells using *RPB1-TAP* tagged strains. The polyubiquitylation level of the purified subunit was then assessed by western blotting with an anti-ubiquitin antibody. As shown in Figure 21B, treatment with 6AU resulted in a significant increase in the polyubiquitylation of Rpb1.

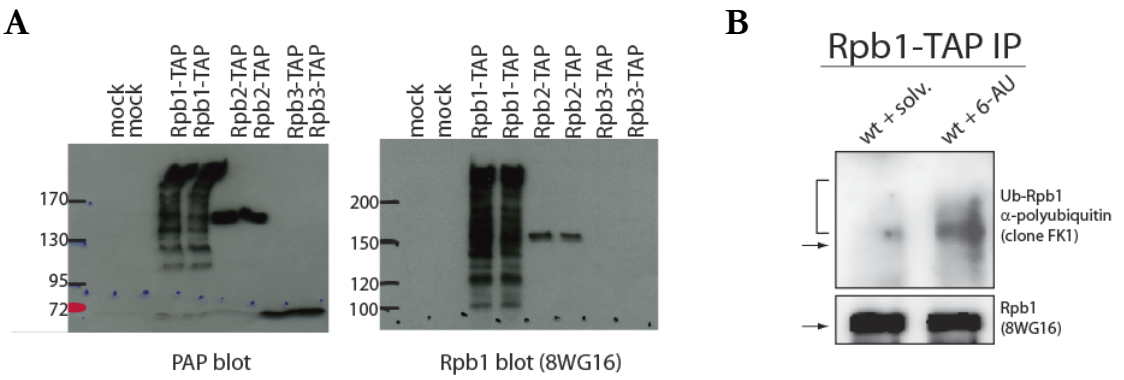

Figure 21 | Polyubiquitylation of Rpb1 is increased upon treatment with 6AU.

A, RNAPII was purified under denaturing conditions using *RPB1*-, *RPB2*- and *RPB3*-TAP strains. Specific purification of polymerase subunits was assessed by western blotting with a PAP and an Rpb1 (8WG16) antibody. Rpb2- and Rpb3-TAP purification did not co-precipitate the Rpb1 subunit indicating that with this method single RNAPII subunits can be purified. The band observed with the 8WG16 antibody in the Rpb2-TAP is a result of the recognition of the TAP-tag by the secondary a-mouse IgG antibody used. **B,** Rpb1 was purified under denaturing conditions as in A from an *RPB1*-TAP strain. Polyubiquitylation of Rpb1 was assessed by western blotting using a-ubiquitin specific antibody (upper panel). The approximate positions of Rpb1 (arrow) and polyubiquitylated Rpb1 (brackets) are indicated.

Taken together, impairment of transcription elongation leads to increased polyubiquitylation of Rpb1 of potentially transcriptionally stalled RNAPII.

2.4.2 A slower polymerizing form of RNAPII has decreased Rpb1 polyubiquitylation.

Two point mutations in the *RPB1* gene, *rpb1-N488D* and *rpb1-E1103G* result in a slower or faster polymerization rate of RNAPII, respectively (Malagon, Kireeva et al. 2006). The properties of the two mutants are summarized in Figure 22A. Both of the mutants are sensitive to 6AU, suggesting the involvement of the affected amino acid in transcription elongation. However, the slower polymerizing *rpb1-N488D* mutant is not synthetic lethal with *DST1* and is thus probably less prone to transcriptional stalling whereas the faster polymerizing *rpb1-E1103G* mutant is synthetic lethal with *DST1* and it was shown to result in an error-prone RNAPII (Walmacq, Kireeva et al. 2009).

A

Comparison of RNAPII mutants	
rpb1-N488D	*rpb1-E1103G*
Slow polymerization	Fast polymerization
Highly conserved	Highly conserved
Higher sensitivity to 6AU	Lower sensitivity to 6AU
Induction of IMD2 expression ↳ amino acid starvation	Not
Synthetic lethal with Soh1	Not
Not	Synthetic lethal with Dst1, Spt4

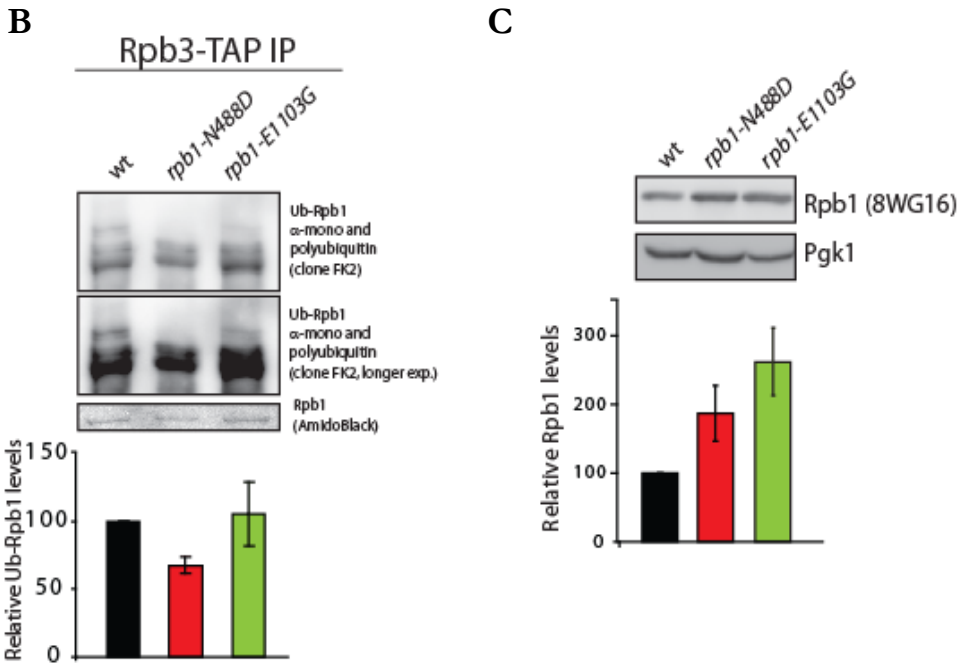

Figure 22 | Slower polymerizing RNAPII results in decreased polyubiquitylation of Rpb1.

A, Table summarizing the properties of the two point mutations in the *RPB1* gene that alter the polymerization rate of RNAPII. **B,** RNAPII was purified from the corresponding yeast strains using Rpb3-TAP. Experiment as in Figure 20. Columns and error bars represent the mean ± standard deviation from 3 independent experiments. **C,** Total cellular Rpb1 levels are increased in the polymerizing mutants. Experiment as in Figure 12. Columns and error bars represent the mean ± standard deviation from 3 independent experiments

Interestingly, *rpb1-N488D* had lower Rpb1 polyubiquitylation levels compared to the wild-type and the *rpb1-E1103G* mutant enzyme (Figure 22B). However, the total cellular levels of Rpb1 in those mutants are increased (Figure 22C). Since both of the mutations are affecting the subunit, the increased protein levels could be a result of the over-production of mutated *RPB1* mRNA.

Taken together, the above experiment indicates that Rpb1 polyubiquitylation is caused by transcriptional stalling in wild-type cells.

2.4.3 The 26S proteasome degrades Rpb1 of transcriptionally stalled RNAPII probably at the site of transcription

The polyubiquitylated Rpb1 is most likely a substrate of the 26S proteasome. Thus, the potential association of the proteasome with RNAPII was examined by co-immunoprecipitation experiments. RNAPII was purified from wild type and the four transcription elongation mutants and the samples were then subjected to western blotting using an antibody recognizing the 20S catalytic subunits of the 26S proteasome. As shown in Figure 23, RNAPII associated with the 20S proteolytic core of the proteasome in wild-type cells (compare first two lanes). This suggests that in wild-type cells the proteasome is physically associated with the polymerase to possibly degrade it upon transcriptional impairment. Importantly, association of RNAPII with the proteasome increased about two-fold when transcription elongation is impaired (Figure 23, lower pannel).

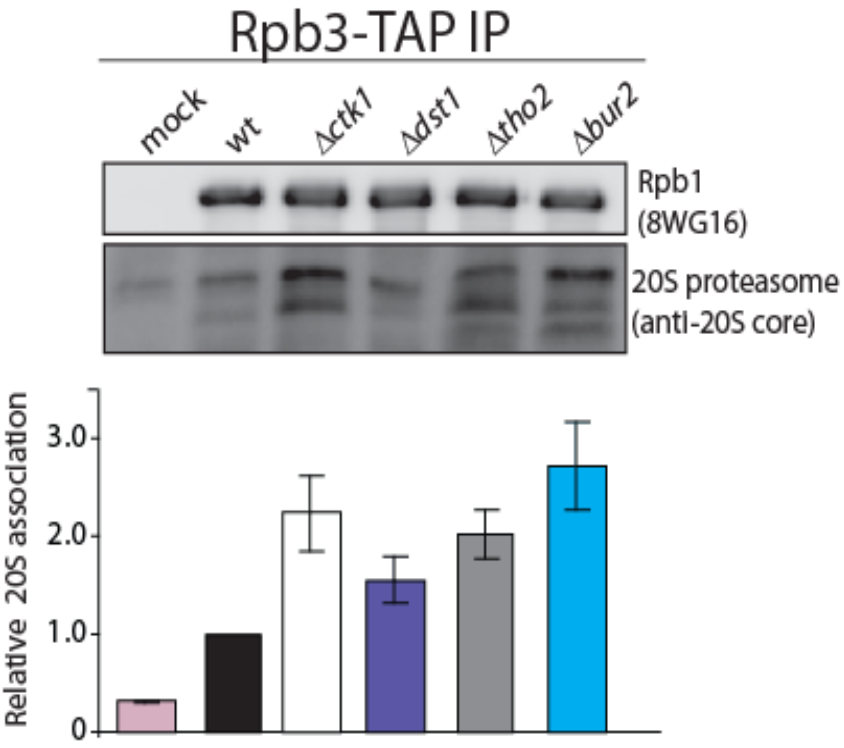

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Figure 23 | Association of RNAPII with the 20S catalytic proteasome is increased in *Δctk1*, *Δdst1*, *Δtho2*, and *Δbur2* cells.

RNAPII was purified using an *RPB3-TAP* strain. Levels of Rpb1 and co-purifying proteasome were detected with 8WG16 and anti-20S core anti-bodies, respectively. Columns and error bars represent the mean ± standard deviation from 4 independent experiments.

If degradation of Rpb1 of transcriptionally stalled RNAPII complexes takes place at the site of stalling, one would expect that the proteasome is recruited to the site of transcription. In order to test this, ChIP experiments were performed with four subunits of the 20S core of the proteasome (Pre1, Pre2, Pre4, and Pup1) since the 19S regulatory particle has been implicated in transcription elongation independently of degradation (Kodadek 2010). Recruitment of the 20S core was assessed in three exemplary genes, *ADH1*, *ACT1*, and *PMA1*. In the transcription elongation mutants, RNAPII occupancy decreases about 2-fold compared to wild-type (Figure 11B) most likely reflecting the decrease in Rpb1 levels (Figure 12A). Importantly, recruitment of the proteasome relative to RNAPII is increased, especially to the middle (M) and 3' region of the *ADH1* gene and the middle (M) regions of *ACT1* and *PMA1* as expected for impaired transcription elongation (Figure 24A-D). This distribution pattern indicates elongation dependent recruitment of the proteasome. Consistently, elongating RNAPII complexes are the preferred substrate for degradation (Mitsui and Sharp 1999; Arima, Nitta et al. 2005; Somesh, Reid et al. 2005; Somesh, Sigurdsson et al. 2007).

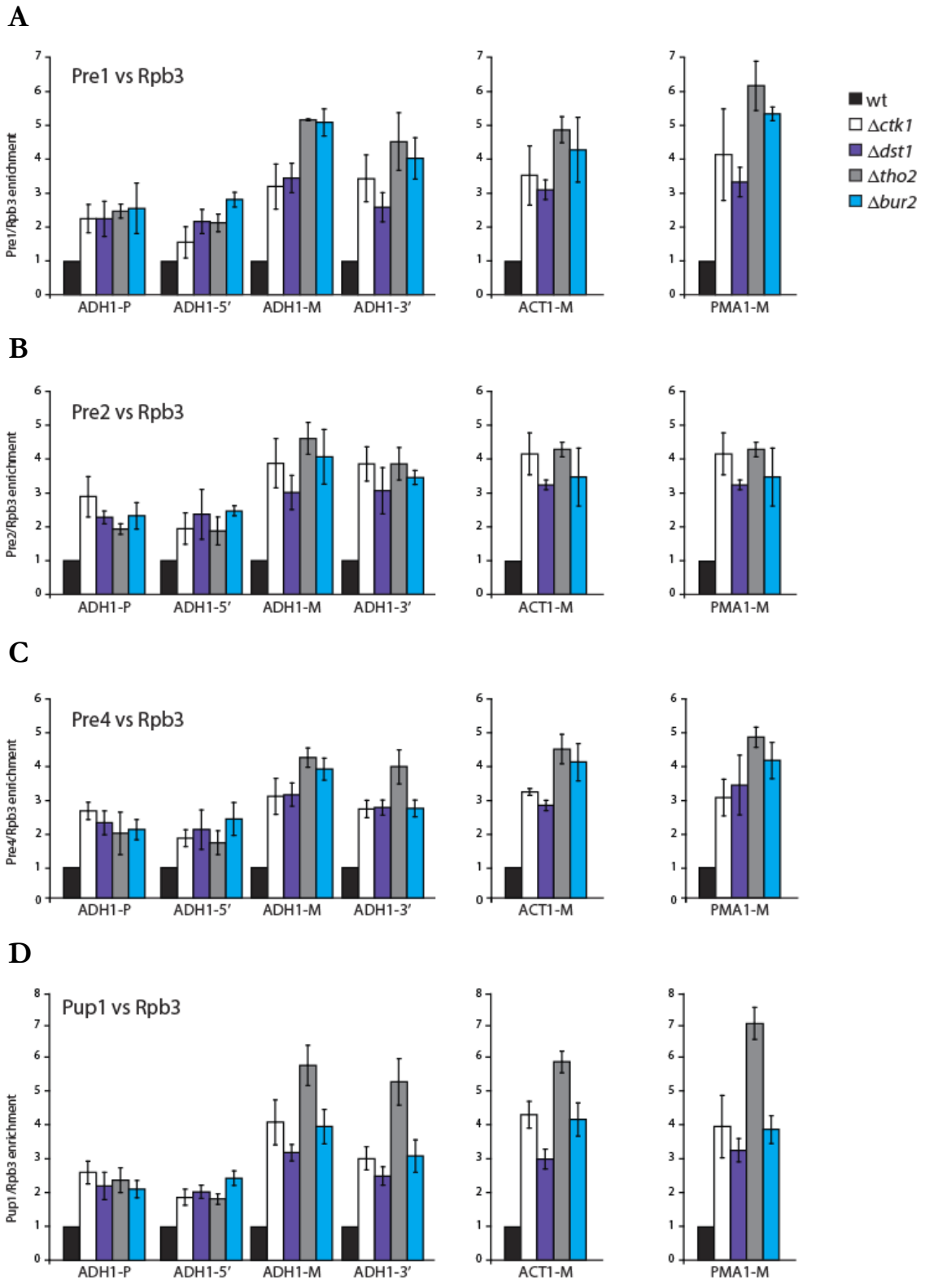

Figure 24 | The proteasome is recruited to the middle and 3' region of actively transcribed genes in *Δctk1*, *Δdst1*, *Δtho2*, and *Δbur2* cells.

ChIP experiments were performed with *PRE1-*, *PRE2-*, *PRE4-*, and *PUP1-TAP* strains. **A-D,** The enrichment of Pre1 (**A**), Pre2 (**B**), Pre4 (**C**) and Pup1 (**D**) relative to Rpb3 in wt cells was set to one. Enrichment of proteasomal subunits on these genes was calculated as in Figure 11. Columns and error bars represent the mean ± standard deviation from 3 independent experiments.

2.5 SUBUNITS OF THE 26S PROTEASOME INTERACT GENETICALLY WITH THE TRANSCRIPTION ELONGATION FACTORS

The physical or functional interaction between two proteins can be indicated by a synthetic lethality phenotype. In general, two alleles are synthetically lethal if the combination of otherwise viable mutations in these genes leads to cell death (Figure 25).

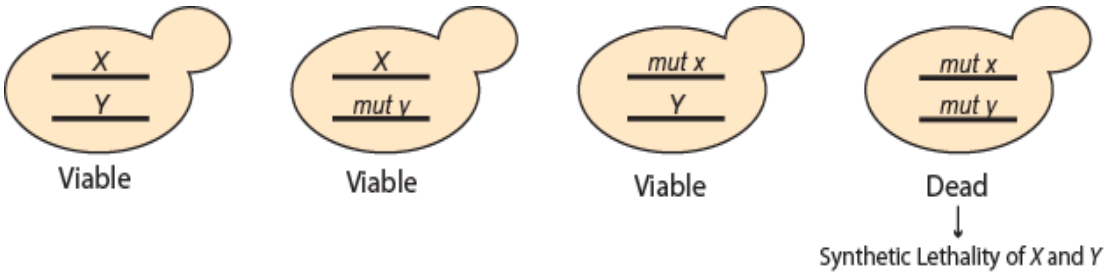

Figure 25 | Principle of Synthetic Lethality.

When the combination of otherwise viable gene mutations results in the death of the yeast cells, then the two genes are synthetically lethal

For the proteasome and the transcription elongation factors a synthetic lethality phenotype would reflect the need of proper proteasomal function for the removal of transcriptionally stalled RNAPII complexes. This means: if in the transcription elongation mutants the rescue mechanism for the degradation of stalled Rpb1 is impaired then the cells are not able to cope with increased transcriptional stalling. This inability would prevent further transcription and subsequently the cells could no longer survive. To test this, temperature-sensitive (ts) mutations of two proteasomal proteins, Pre1 and Cim3, were combined with the deletions of the four transcription elongation mutants. Pre1 is a subunit of the 20S catalytic core and Cim3 is an ATPase of the 19S regulatory particle of the proteasome. The *pre1-1* and the *cim3-1* alleles of the proteasome were found to be synthetic lethal with *CTK1*, *THO2* and *BUR2* and in the presence of 6AU with *DST1* (Figure 26). This result

further supports the finding that the 26S proteasome is responsible for Rpb1 degradation in the transcription elongation mutants.

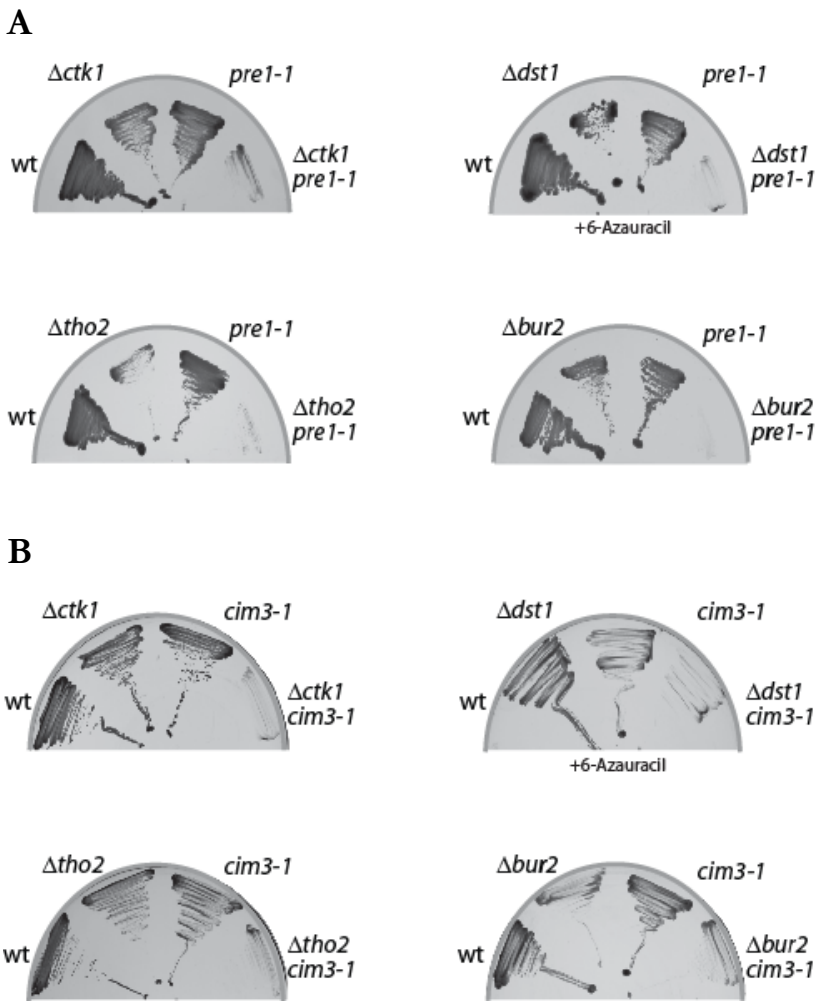

Figure 26 | *PRE1* and *CIM3* are synthetic lethal with *CTK1*, *DST1*, *THO2* and *BUR2*.

The deletion mutants of the four transcription elongation factors were combined with the temperature sensitive alleles of *PRE1* (shown in **A**) and *CIM3* (shown in **B**), *pre1-1* and *cim3-1* respectively. The single mutations were viable but showed a growth defect. However, the double mutant strains were dead showing the genetic interaction of the genes. For *Δdst1*, 50 µg/mL of 6AU was added to the plates.

Moreover, TAP tagging the 19S components *CIM3* and *CIM5* in the transcription elongation mutants resulted in an inviable phenotype, except for the case of Dst1 (Figure 27). This genetic interaction is consistent with previous findings that the 19S regulatory particle of the proteasome is needed for efficient transcription elongation.

Finally, temperature-sensitive mutants of the proteasome were tested for their sensitivity to the transcription drugs 6AU and Mycophenolic Acid (MPA), both affecting the nucleotide pool inside the cell (Exinger and Lacroute 1992). Only the *cim3-1* mutant was sensitive to both 6AU (Figure 28A) and MPA (Figure 28B) whereas the rest of the ts mutants tested were not. As a positive control the sensitivity of the transcription elongation mutants was tested under the same growth conditions (Figure 28A and B, lower panels). The sensitivity to both of the drugs was more pronounced for the *Δdst1* cells.

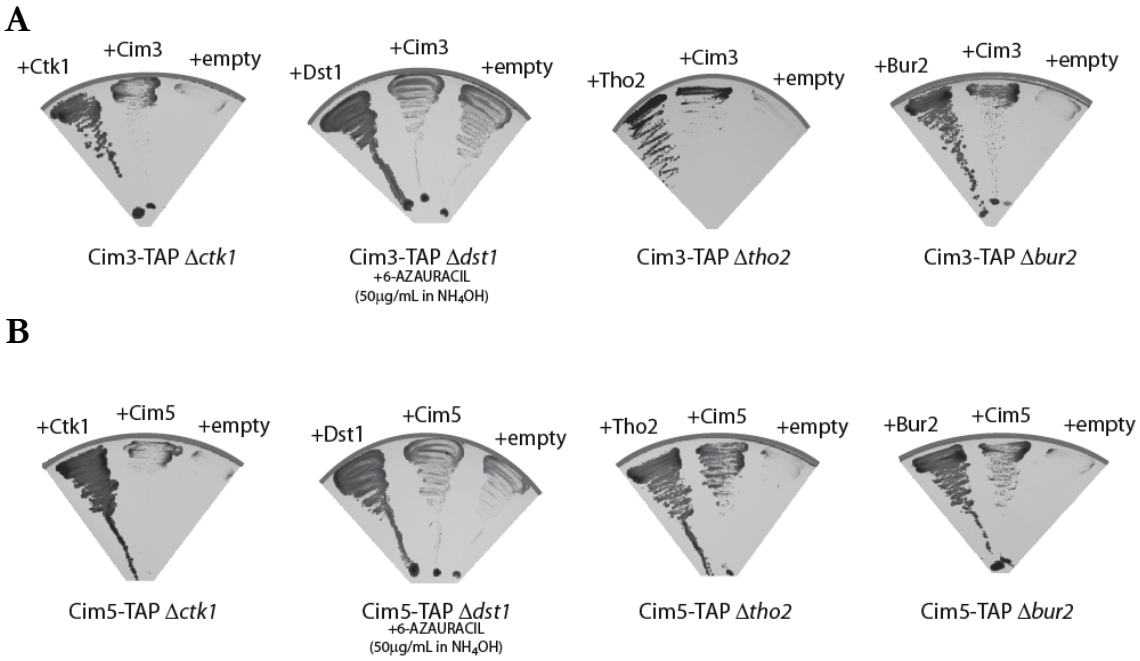

Figure 27 | *CIM3*- and *CIM5-TAP* are synthetic lethal with *CTK1*, *THO2* and *BUR2* and are not synthetic lethal not with *DST1*.

The deletion mutants of the four transcription elongation factors were combined with TAP-tagged versions of *CIM3* and *CIM5*. The combination of the three transcription factor deletions and the tagging of the 19S component resulted in a synthetic lethal phenotype. For *Δdst1*, 50 µg/mL of 6AU was added to the plates.

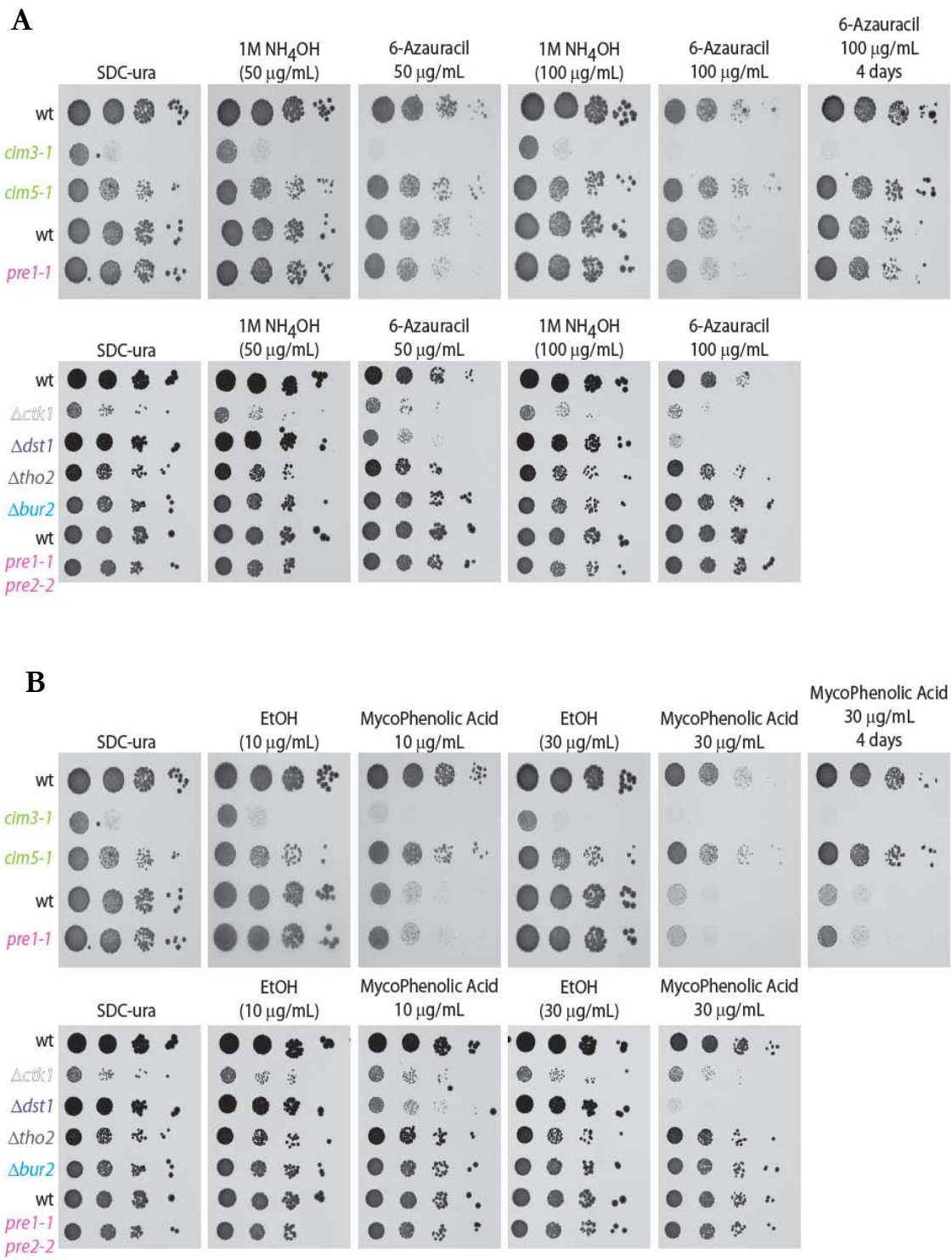

Figure 28 | Sensitivity of proteasome and transcription elongation factor mutants to the transcription elongation drugs 6-Azauracil (6AU) and Mycophenolic Acid (MPA).

10-fold serial dilutions of the indicated yeast strains were spotted onto SDC-ura plates supplemented with 6AU or MPA. The plates were incubated at 30°C for 3 or where indicated 4 days. The transcription elongation mutants *Δctk1, Δdst1, Δtho2* and *Δbur2* and the proteasome mutant *cim3-1* were sensitive to both transcription elongation drugs.

2.6 MOLECULAR MECHANISM FOR THE POLYUBIQUITYLATION OF TRANSCRIPTIONALLY STALLED RNAPII.

The above experiments showed that upon transcriptional stalling there is polyubiquitylation of Rpb1. In general, polyubiquitylation is mediated by a series of ubiquitin modifying enzymes which are responsible for: (i) the production of a ubiquitin-lysine specific polyubiquitin chain and (ii) the attachment of this chain in an internal lysine residue of the protein targeted for degradation.

2.6.1 The polyubiquitin chains on Rpb1 are mainly K63-linked and are required for degradation.

There are two major types of polyubiquitin chains formed. The one is formed through Lys48 of ubiquitin and it is the one present in most proteins which are targeted for degradation. The second one is formed through Lys63 of ubiquitin and has mainly regulatory purposes although recently it was shown to be involved in the degradation of proteins as well (Figure 6). To determine the nature of the polyubiquitin chain attached to Rpb1, antibodies directed against K48- (Apu2.07) or K63- (Apu3.A8) ubiquitin lysine-specific chains were used (Newton, Matsumoto et al. 2008). To test the specificity of these antibodies chemically synthesized polyubiquitin chains of either type were subjected to western blot. Both antibodies recognized with a high specificity and with no cross-reaction only their type of lysine-specific chains (Figure 29). The high specificity of these antibodies allowed assessing the nature of the polyubiquitin chain attached on transcriptionally stalled Rpb1. To that end RNAPII was purified from wild-type and the four transcription elongation mutants and the samples were subjected to western blot with the lysine specific antibodies. As shown in Figure 30, the polyubiquitin chains attached to Rpb1 in the four transcription elongation mutants are mainly formed through K63 of ubiquitin. Consistently, polyubiquitin chains of transcriptionally stalled mammalian RNAPII formed *in vitro* are K63-linked (Lee and Sharp 2004).

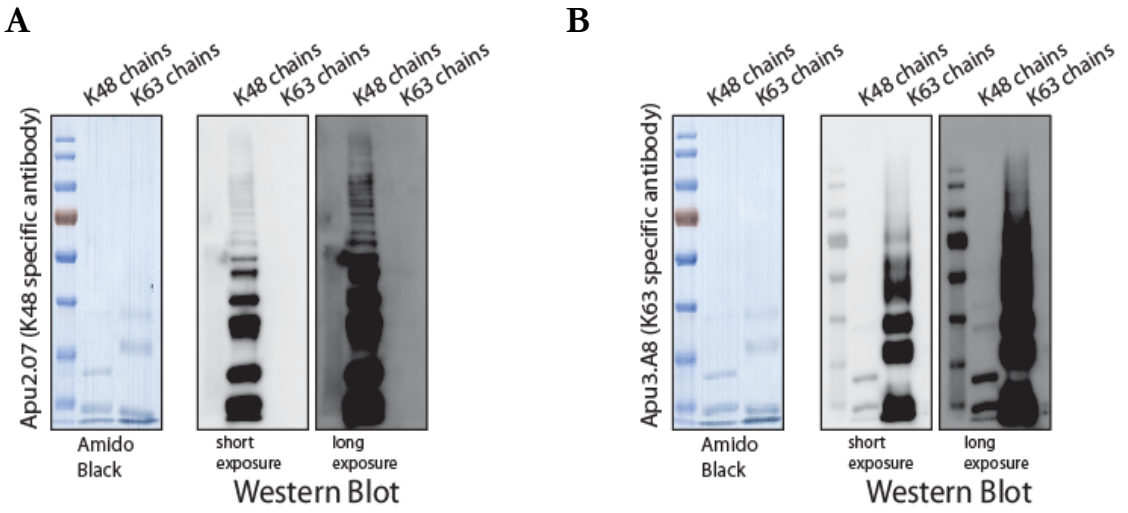

Figure 29 | The Apu2.07 and Apu3.A8 antibodies specifically recognize only K48- or K63-linked polyubiquitin chains respectively.

Chemically synthesized K48- or K63- polyubiquitin chains (Sigma) were subjected to western blotting with the Apu2.07 and Apu3.A8 antibodies. **A,** The Apu2.07 only reacted with the K48 chains and **B,** the Apu3.A8 only with the K63 chains, showing the high specificity of these antibodies. Subsequent to exposure, the membranes were stained with AmidoBlack for detecting loading of proteins.

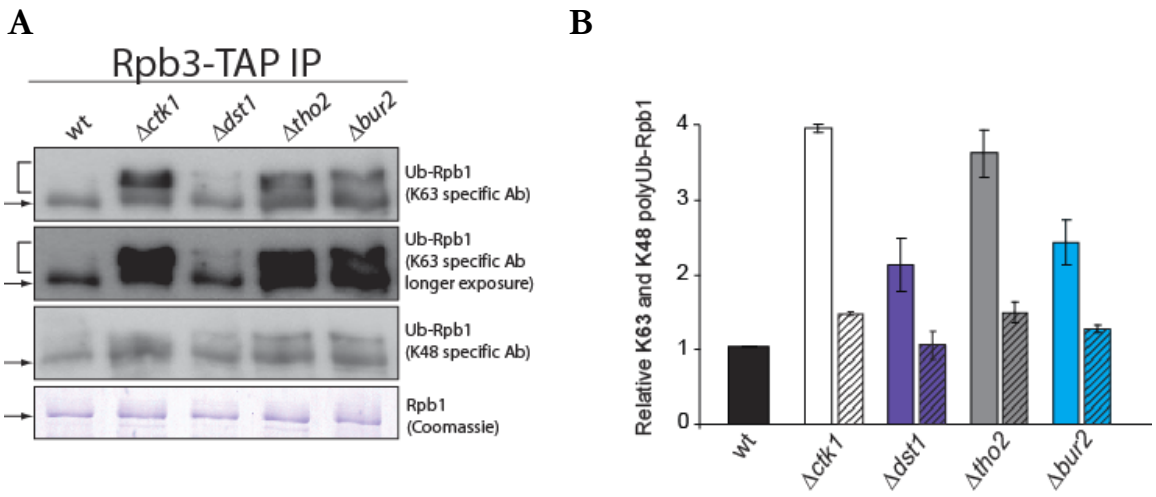

Figure 30 | Polyubiquitin chains formed on Rpb1 in *Δctk1*, *Δdst1*, *Δtho2* and *Δbur2* cells are mainly K63-linked.

A, RNAPII was purified from the indicated strains. Experiment as in Figure 20. The nature of polyubiquitin chains was assessed by western blotting using K48- and K63-ubiquitin specific antibodies and **B,** Quantification of relative levels of polyubiquitylated RNAPII. Columns and error bars represent the mean ± standard deviation from 4 independent experiments.

There is also a small amount of K48-linked chains observed indicating that there might be two pools of polyubiquitylated Rpb1 upon transcriptional stalling (Figure 30, lower pannel). Alternatively, the observed K63-linked polyubiquitin chains could be remodelled by deubiquitylases and a K48-linked chain added by a yet unknown E3 ligase for degradation of Rpb1 (also see below), which would be consistent with the observed small increase in K48-linked chains (Figure 30, lower pannel).

Since proteins carrying K63-linked polyubiquitin chains can be degraded by the proteasome *in vitro* and *in vivo* (Kirkpatrick, Hathaway et al. 2006; Kim, Kim et al. 2007; Saeki, Kudo et al. 2009), K63-polyubiquitylated Rpb1 of transcriptionally stalled RNAPII might be the substrate for degradation. Consistently, K63-polyubiquitylated Rpb1 is readily degraded by the 26S proteasome *in vitro* whereas nonphysiological polyubiquitylated substrates (e.g., GFP) are not (Saeki, Kudo et al. 2009). In order to test directly whether K63-linked polyubiquitin chains are essential for degradation of Rpb1 *in vivo*, the total Rpb1 levels in an ubi-K63R strain were assessed. Deletion of *DST1* leads to a decrease in total Rpb1 levels to about 70% compared to the corresponding wild-type strain (Figure 12A and 31). This decrease in Rpb1 levels does not occur when transcription elongation is impaired by deletion of *DST1* in the K63R background (Figure 31) indicating that K63-linked polyubiquitin chains are needed *in vivo* for Rpb1 degradation caused by transcriptional stalling.

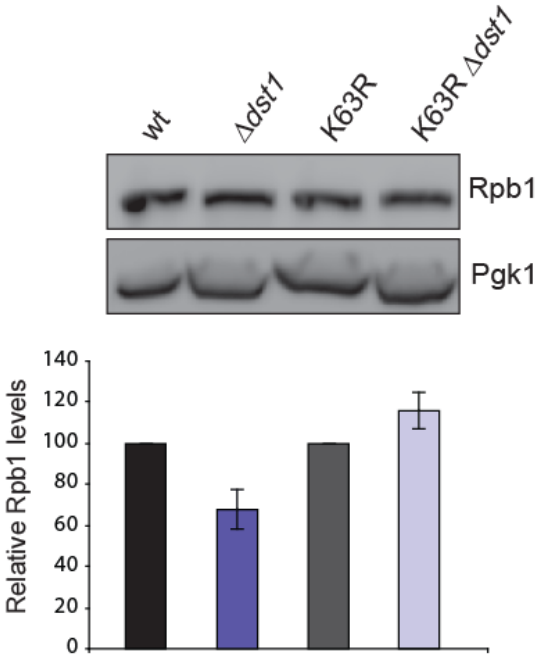

Figure 31 | K63-linked linked chains on Rpb1 are required for its degradation *in vivo*.
Western blot analysis of total Rpb1 levels in the indicated strains. Rpb1 levels in wild-type and K63R strains were set to 100. Experiment as in Figure 12. Columns and error bars represent the mean ± standard deviation from 4 independent experiments (bottom panels).

Taken together, the polyubiquitin chains attached to Rpb1 are formed mainly through K63 of ubiquitin and these chains are probably needed *in vivo* for the transcriptional stalling-dependent degradation of RNAPII.

2.6.2 The polyubiquitin chain is attached on K330 and K695 of Rpb1.

The polyubiquitin chain formed is attached to a lysine residue of the substrate targeted for degradation. However, there are several proteins known in which more than one lysine are needed for the polyubiquitylation of the protein (Peng, Schwartz et al. 2003). Rpb1 is a 190 kDa protein with 93 lysine residues that are located throughout the surface of the protein (Figure 32, green residues). Upon DNA damage-dependent degradation of RNAPII the lysines of Rpb1 that serve as ubiquitylation sites have been mapped (see Introduction 1.8).

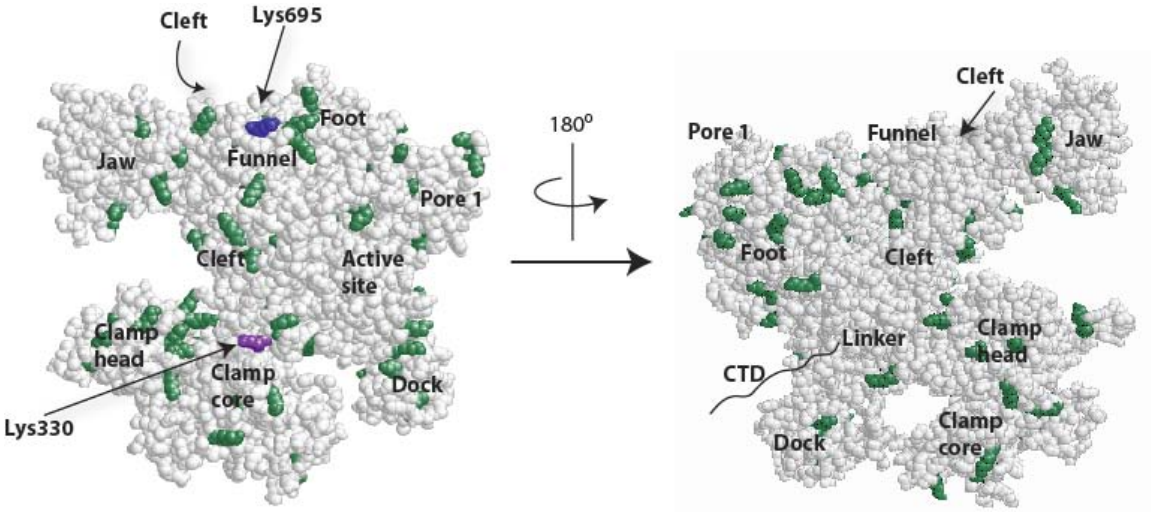

Figure 32 | Localization of Lysine residues of Rpb1.

Schematic representation of the localization of the 93 lysine residues (green) of Rpb1 in respect to the different domains of the protein. The characterized ubiquitylation sites K330 and K695 are colored magenta and blue respectively.

The first of them is K330 (Figure 32, magenta lysine) located in a protein domain that is unordered in free RNAPII, but ordered in the elongating form. The other one is K695 (Figure 32, blue lysine) which is located >125 Ångstroms away from the first one. Both of these residues were shown to be involved in the polyubiquitylation of Rpb1 as mutation of either site affects ubiquitylation of the other, *in vitro* and *in vivo*. To determine whether these residues are involved in the transcriptional stalling-dependent degradation of Rpb1, the *rpb1-K330R* and *rpb1-K695R* mutations were introduced in wild-type cells and the four transcription elongation mutants. The Rpb1 mutations in combination with the transcription elongation mutants did not further affect the growth phenotype of the cells (Figure 33A).

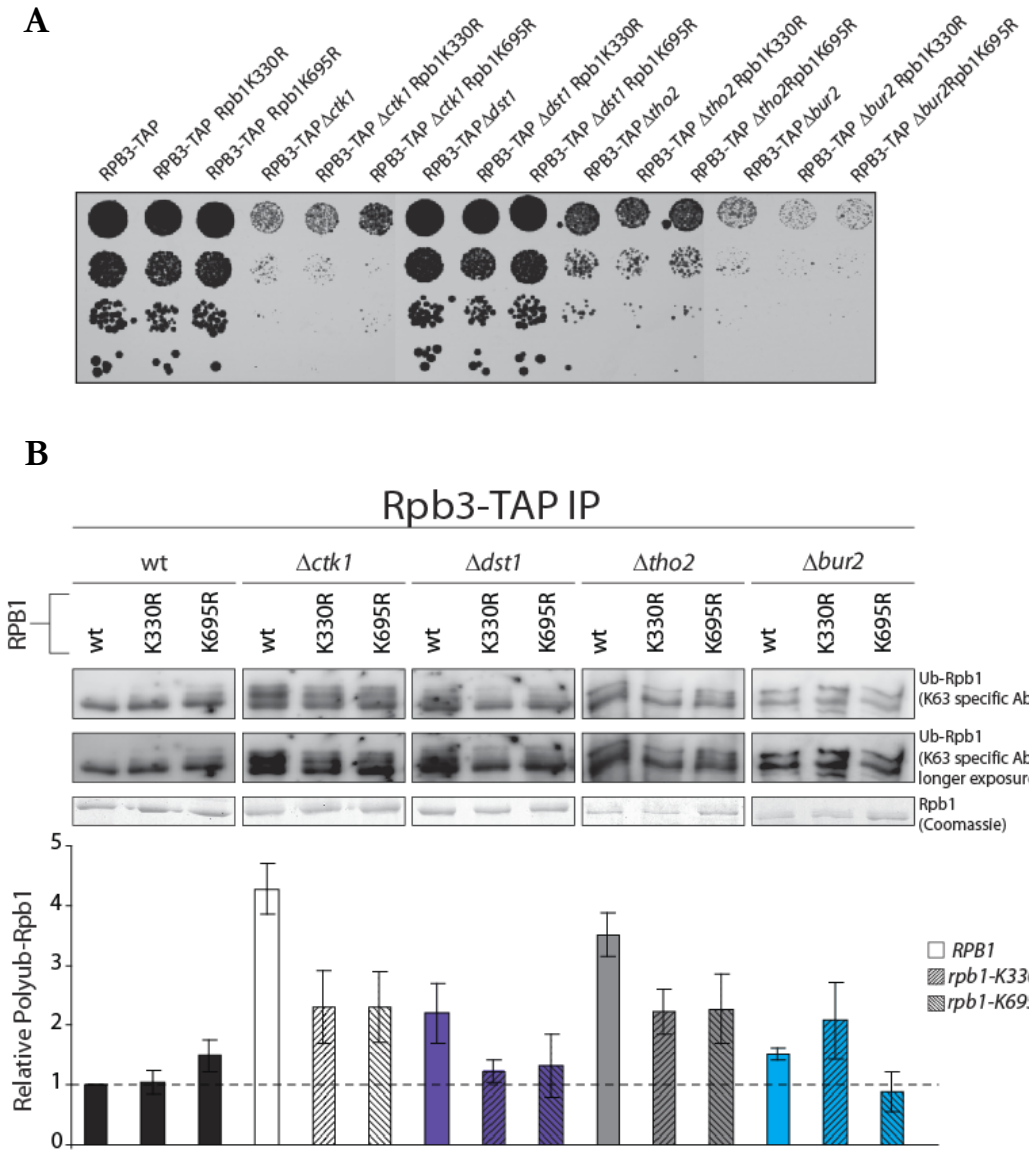

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Figure 33 | The polyubiquitin chain is attached on K330 and K695 of Rpb1.

A, Growth analysis of the combination of the transcription elongation mutants with the *RPB1* lysine mutants showed no synthetic lethality phenotype. **B,** RNAPII was purified from the indicated strains, experiment as in Figure 20. Polyubiquitylation of Rpb1 was assessed by western blotting with the K63-linkage specific antibody. The *rpb1-K330R* and *rpb1-K695R* mutations in Δ*ctk1,* Δ*dst1,* Δ*tho2,* and Δ*bur2* cells resulted in the reduction of polyubiquitylated Rpb1 compared to the one observed in each deletion mutant with wild-type Rpb1. Columns and error bars represent the mean ± standard deviation from 3 independent experiments.

RNAPII was purified from the cells and the samples were then subjected to western blotting using anti-ubiquitin antibodies to detect the polyubiquitylation levels of Rpb1. In wild-type cells both Rpb1 mutations did not affect the basal polyubiquitylation of the protein (Figure 33B, first panel in right).

However, upon transcription elongation impairment either the *rpb1-K330R* or the *rpb1-K695R* mutation resulted in reduced levels of polyubiquitylated Rpb1 compared to the one observed in each transcription elongation mutant with wild-type Rpb1 (Figure 33B). Nevertheless, since Rpb1 has several lysine residues one can not exclude the presence of a third ubiquitylation site on Rpb1. The ideal experiment with both of the identified sites mutated, in order to potentially abolish Rpb1 ubiquitylation completely, is not possible since the K330R and K695R mutations are synthetic lethal. This synthetic lethality phenotype supports the idea that the two sites perform overlapping roles in RNAPII biology.

Taken together, in the transcriptional stalling-dependent degradation of RNAPII the polyubiquitin chain is attached to two sites on Rpb1: K330 and K695.

2.6.3 Ubiquitin modifying enzymes involved in polyubiquitylation of Rpb1.

Ubiquitylation requires the cooperation of three enzymes: the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin ligase (E3) (see

Introduction 1.7.1). There is only one E1 in eukaryotic cells (Uba1 in *S.cerevisiae*), but several E2s and a lot more E3s. This study aimed to uncover the E2 and E3 enzymes responsible for the polyubiquitylation of Rpb1 of transcriptionally stalled RNAPII. To test the function of the candidate ubiquitin modifying enzymes in the transcriptional stalling-dependent pathway the corresponding genes were deleted or mutated in the *Δdst1* genetic background. The *Δdst1* mutant was selected among the other three transcription elongation mutants since it is the one exhibiting wild-type like growth (Figure 10) and – more importantly– it is not synthetic lethal with any of the enzymes tested (Figures 34A, 35 and (Lee, Yu et al. 2001; Woudstra, Gilbert et al. 2002).

2.6.3.1 Ubc4 and Ubc5 are the E2 conjugating enzymes.

In *S.cerevisiae*, there are two housekeeping E2 enzymes, Ubc4 and Ubc5, with redundant function. Although these proteins are encoded by very similar genes (97% similar at the amino acid level) the expression of Ubc4 is much higher that the one of Ubc5. Deletion of Ubc4 leads to a mild growth defect and a weak sensitivity to diverse environmental stresses. In contrast, loss of Ubc5 has no effect probably due to the presence of high levels of Ubc4 (Seufert and Jentsch 1990) (Figure 34A). It was shown that the E2s Ubc4 and Ubc5 are needed for polyubiquitylation of Rpb1 in response to transcriptional stalling but also in response to DNA damage (Somesh, Reid et al. 2005).

To corroborate the function of Ubc4 and Ubc5 in polyubiquitylation of Rpb1 in the transcription dependent pathway, RNAPII was purified from strains lacking either Dst1 or the E2 enzyme or the combination of both. The samples were then subjected to western blotting using anti-ubiquitin antibodies. Ubc4 and Ubc5 are indeed involved in the polyubiquitylation of Rpb1: in the double deletion mutants (*Δdst1 Δubc4; Δdst1 Δubc5*) there was approximately 50% less polyubiquitylated Rpb1 compared to the one observed in the *Δdst1* cells (Figure 34B). This is most likely due to the overlapping roles of the two enzymes.

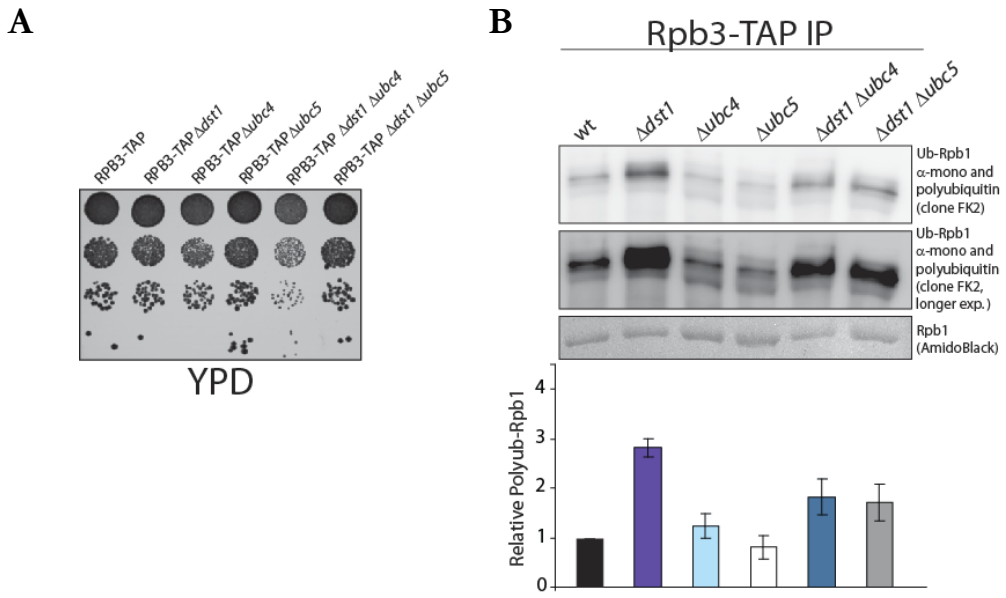


Figure 34 | The E2 enzymes Ubc4 and Ubc5 are responsible for the polyubiquitylation of transcriptionally stalled Rpb1.

A, Growth analysis of the single and double deletion mutants of Dst1, Ubc4 and Ubc5 showed no synthetic lethality phenotype. **B,** RNAPII was purified from the indicated strains. Experiment as in Figure 20. Deleting the E2s *UBC4* and *UBC5* in Δ*dst1* cells resulted in the reduction of polyubiquitylated Rpb1 compared to the one observed in *Δdst1*. Columns and error bars represent the mean ± standard deviation from 3 independent experiments.

2.6.3.2 Rsp5 but not Elc1 is the E3 ligase.

For the efficient ubiquitylation of a substrate the function of the E2 enzyme is followed by the subsequent function of an E3 ligase. The E2 and the E3 form a transient complex, which recognizes and ubiquitylates the protein substrate. Upon DNA damage there are two E3s that have been identified to ubiquitylate Rpb1 (see Introduction 1.8). These are the HECT domain ligase Rsp5 (NEDD4 in humans) and the RING finger domain-containing Elc1/Cul3 complex (Elongin/ /Rbx1/Cullin 5 complex in humans). It was recently shown that upon DNA damage the two enzymes function sequentially, with Rsp5 mono-ubiquitylating Rpb1 and Elc1 adding a K48-linked polyubiquitin chain. In

addition, Rsp5 produces non-functional K63-linked polyubiquitin chains (Harreman, Taschner et al. 2009). So, these proteins were tested for a function in the transcriptional stalling-dependent pathway using one of the transcription elongation mutants, *Δdst1*. The polyubiquitylation of Rpb1 was assessed in either the single or the double mutant strains (Figure 35A and B).

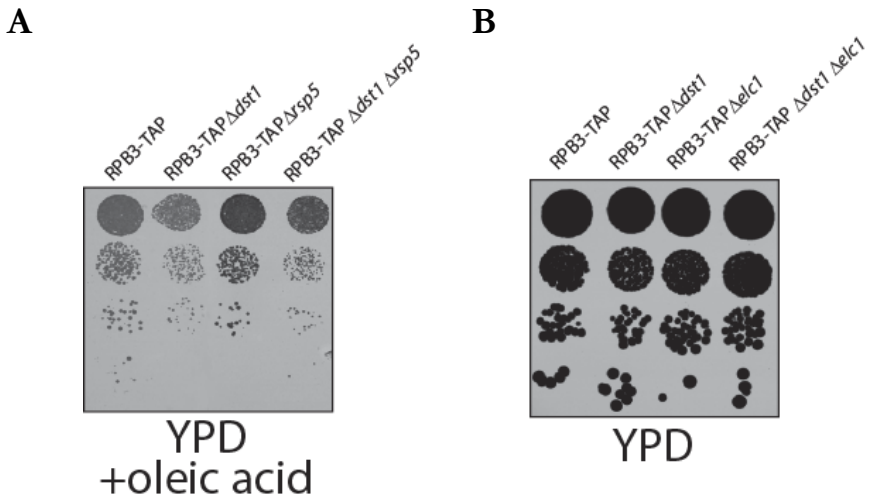


Figure 35 | The E3 enzymes Rsp5 and Elc1 are not synthetic lethal with Dst1.
A, Growth analysis of the single and double deletion mutants of *DST1* and *RSP5*. To rescue the lethality of the *Δrsp5* mutation, the medium was supplemented with 0.2% NP40 and 2mM oleic acid. **B,** Growth analysis of the single and double deletion mutants of *DST1* and *ELC1*.

Interestingly, the additional deletion of *RSP5* in the *Δdst1* background resulted in reduced levels of polyubiquitylated Rpb1 compared to the single *Δdst1* cells (Figure 36A). So, the E3 Rsp5 polyubiquitylates Rpb1 in response to transcriptional stalling. Importantly, however, the combinational deletion of *ELC1* and *DST1* resulted in even higher levels of polyubiquitylated Rpb1 (Figure 36B). This shows that the E3 Elc1 is not required for polyubiquitylation of Rpb1.

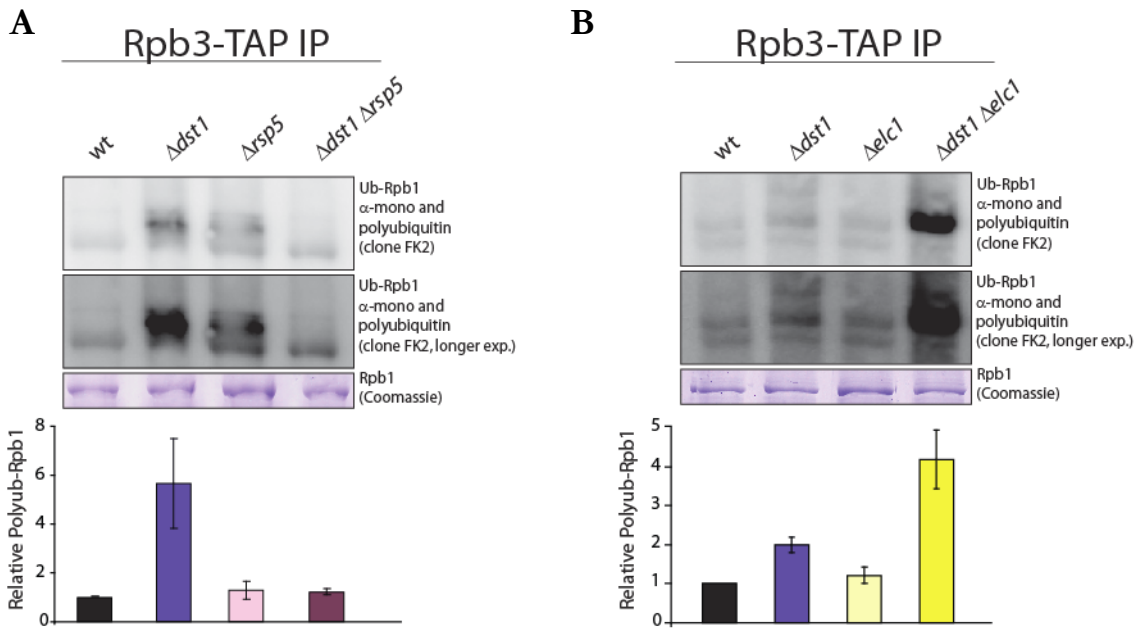


Figure 36 | Rsp5 and not Elc1 is the responsible E3 ligase.

RNAPII was purified from the indicated strains. Experiment as in Figure 20. **A,** Deleting the E3 Rsp5 and in Δ*dst1* cells resulted in the reduction of polyubiquitylated Rpb1 compared to the one observed in *Δdst1* (cells were grown on oleic acid; see Figure 31A). **B,** Deleting the E3 Elc1 and in Δ*dst1* cells resulted in a significant increase of polyubiquitylated Rpb1 compared to the one observed in *Δdst1*. Columns and error bars represent the mean ± standard deviation from 3 independent experiments.

This increase might be explained by a function of Elc1 in promoting efficient transcription elongation ((LeJeune, Chen et al. 2009) and references therein). So, in contrast to the DNA damage-dependent pathway, Rsp5 but not Elc1 is required for polyubiquitylation of Rpb1 upon transcriptional stalling.

2.6.3.3 Investigation of a possible novel E3 ligase.

The polyubiquitin chains on Rpb1 of transcriptionally stalled RNAPII are mainly K63-linked. However, in the four transcription elongation mutants there was also a slight increase in the K48-linked chains observed (Figure 30). These K48-linked chains could be the result from the function of a yet unknown E3 ligase that is required for efficient

degradation of Rpb1. To investigate this, two candidate E3 enzymes were tested for their role in polyubiquitylation of transcriptionally stalled RNAPII. Asr1 is a RING finger ubiquitin-ligase that was selected for analysis since it binds directly to RNAPII via the CTD of Rpb1 (Daulny, Geng et al. 2008). Bre1 is another RING-finger E3 that forms heterodimer with Rad6p to monoubiquitylate histone H2B on K123 and was selected for analysis since it is synthetic lethal with *CTK1* and is needed for efficient transcription (Wood, Schneider et al. 2003; Lee, Shukla et al. 2007; Xiao, Shibata et al. 2007).

However, deletion of both did not affect polyubiquitylation of Rpb1 upon transcriptional stalling (Figure 37). Further analysis of additional E3 ligases is required to potentially identify the enzyme responsible for the K48-linked chains shown in Figure 30.

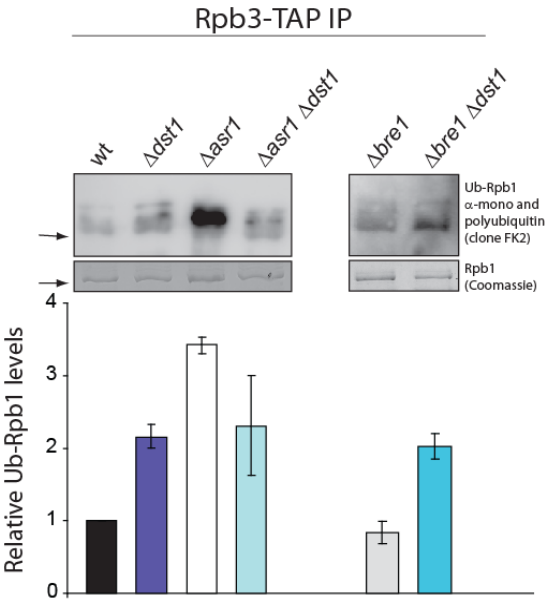


Figure 37 | Asr1 and Bre1 are not required for Rpb1 polyubiquitylation.

RNAPII was purified from the indicated strains. Experiment as in Figure 20. Deleting either Asr1 or Bre1 in the *Δdst1* strain did not affect the levels of polyubiquitylated Rpb1. Columns and error bars represent the mean ± standard deviation from 3 independent experiments

2.6.3.4 Involvement of the ubiquitylation promoting protein Def1 and the TCR factor Rad26.

Upon DNA damage in the transcribed strand the cell attempts to repair the damage by TCR. However, if this fails RNAPII becomes persistently stalled at the site of damage and thus has to be removed to allow continued transcription (see Introduction 1.8). The switch

from repair to degradation is mediated by a complex formation of the TCR protein Rad26 and the ubiquitylation promoting protein Def1.

To test whether these two proteins are involved in the Rpb1 polyubiquitylation upon transcriptional stalling the *DEF1* and *RAD26* genes were deleted in the *Δdst1* genetic background. Consequently, RNAPII was purified from the single and double mutant strains and the samples were subjected to western blotting with anti-ubiquitin antibodies. As shown in Figure 38A, there was a significant reduction in the levels of polyubiquitylated Rpb1 in the *Δdst1 Δdef1* cells as compared to the ones observed in the *Δdst1* cells. Moreover, the single deletion of *DEF1* resulted in a dramatic increase in the polyubiquitylation of Rpb1 (Figure 38A). This observation could reflect another unknown function of Def1 in transcription elongation.

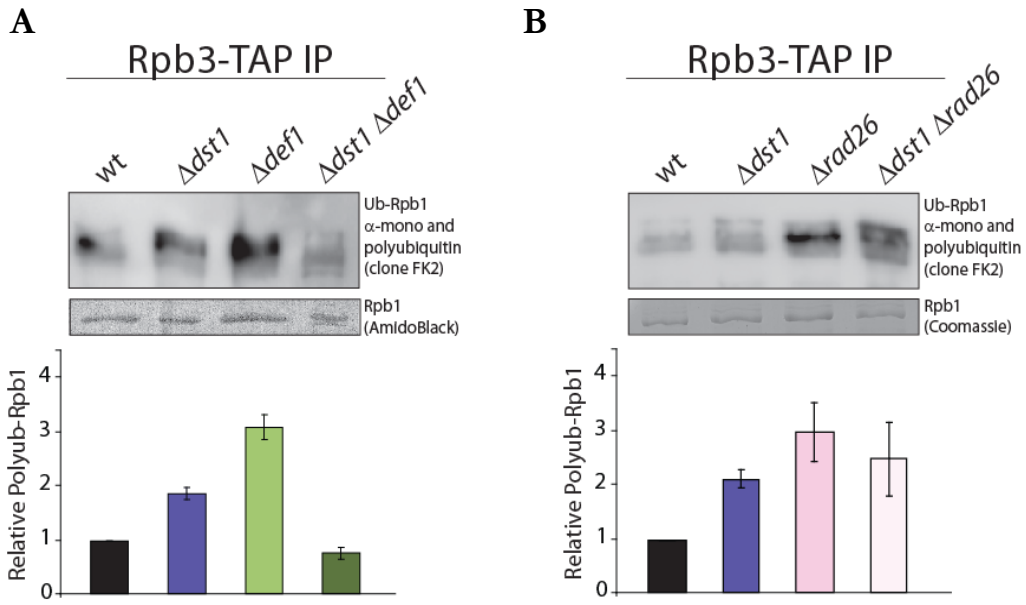


Figure 38 | Def1 and not Rad26 is required for efficient Rpb1 polyubiquitylation.

RNAPII was purified from the indicated strains. Experiment as in Figure 20. **A,** Deleting the ubiquitylation promoting protein Def1 in *Δdst1* cells resulted in the reduction of polyubiquitylated Rpb1 compared to the one observed in *Δdst1*. **B,** Deleting the TCR factor Rad26 in the *Δdst1* strain did not affect the levels of polyubiquitylated Rpb1. Columns and error bars represent the mean ± standard deviation from 3 independent experiments

However, loss of Rad26 function does not affect polyubiquitylation of Rpb1 in the Δ*dst1* background indicating that Rad26 is not involved in the transcription dependent pathway (Figure 38B). The increase in the levels of polyubiquitylated Rpb1 observed in the single *Δrad26* mutated strain might come from the reported inhibitory role of Rad26 in RNAPII ubiquitylation or degradation which enables the transcription block to first be sampled by TCR factors (Woudstra, Gilbert et al. 2002).

2.7 MOLECULAR MECHANISM FOR DE-UBIQUITYLATION OF TRANSCRIPTIONALLY STALLED RNAPII.

After polyubiquitylation of the substrate targeted for degradation, the 26S proteasome is responsible for its proteolysis. However, in the case of transcriptional stalling it is possible that through the function of general transcription elongation factors the stalled complex escapes arrest and is then able to restart transcription. Since RNAPII is an important enzyme a rescue mechanism must exist to prevent unnecessary Rpb1 degradation in complexes that are not stalled any more. So, ‘‘proofreading’’ of Rpb1 ubiquitylation by deubiquitylases (DUBs) is crucial for cell viability. This “proofreading” could serve for delaying RNAPII degradation until transcription factors can help the stalled complex to resume transcription by maintaining short-length polyubiquitin chain. Additionally, deubiquitylation by the DUBs could serve for preventing degradation of a rescued from staling RNAPII complex by cleaving off the polyubiquitin chain from Rpb1 to produce completely deubiquitylated RNAPII. *S.cerevisiae* cells contain 17–20 DUBs, 16 of which belong to the specific ubiquitin protease (UBPs) family. The UBPs antagonize the ubiquitylation of proteins, playing a role analogous to that of the phosphatases in a kinase/phosphatase regulatory pathway (see Introduction 1.7.3).

In the DNA damage-dependent pathway there are two deubiquitylases that have been described to be involved: Ubp2 deubiquitylates excess K63-linked polyubiquitin chains formed by Rsp5, whereas Ubp3 deubiquitylates Rsp5-mediated monoubiquitin and Elc1-

mediated K48-linked polyubiquitin chains from Rpb1 (Kvint, Uhler et al. 2008; Harreman, Taschner et al. 2009). These two proteins along with 3 more enzymes (Ubp6, Ubp10 and Ubp12) were selected based on their sensitivity to 6AU (Kvint, Uhler et al. 2008) to be tested for their possible function in deubiquitylating Rpb1 in response to transcriptional stalling. To this end, yeast strains were created that lacked each of these enzymes either alone or in combination with the deletion of *DST1*. All the double mutants were viable and no additive growth defects were observed (Figure 39A).

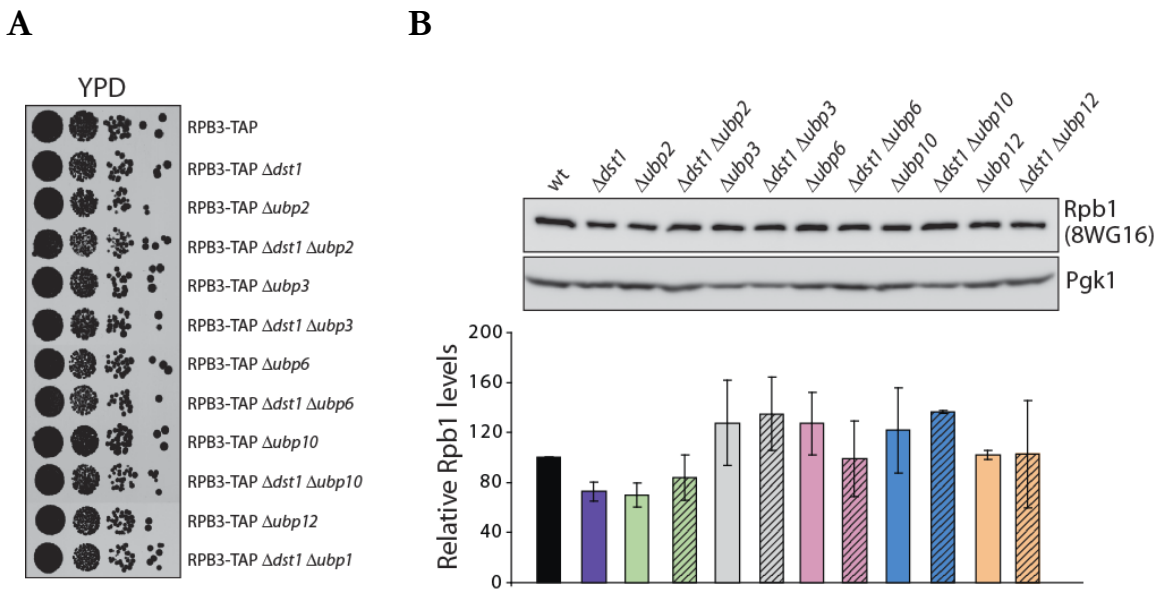


Figure 39 | The UBPs Ubp2, 3, 6, 10,12 do not affect the growth of Δ*dst1* cells and the total cellular levels of Rpb1.

A, Growth analysis of the single and double deletion mutants of *DST1* and the different UBPs. No synthetic lethality phenotype was observed. **B,** Total cellular Rpb1 levels were quantified in the indicated strains. Experiment as in Figure 12. No significant change was observed in the double mutants. Columns and error bars represent the mean ± standard deviation from 3 independent experiments

The UBPs are known to affect the stability of ubiquitylated proteins (Chung and Baek 1999; Wilkinson 2000). To investigate the effect of the deletion of the UBPs in RNAPII levels, the protein levels of Rpb1 were quantified in the single and double deletion strains. As shown in Figure 39B, there was no increased degradation of Rpb1 in any of the UBPs tested. This indicates that deletion of the deubiquitylases does not reduce the half-life of polyubiquitylated Rpb1.

To investigate which of the candidate UBPs is involved in deubiquitylating RNAPII in the transcriptional stalling-dependent pathway the level of polyubiquitylated Rpb1 was assessed in the single and the double deletion mutants. Consistent with a function of Rsp5 in ubiquitylating Rpb1 of transcriptionally stalled RNAPII complexes, Ubp2 is required for deubiquitylation of Rpb1 (Figure 40, compare lanes 2 and 6). However, Ubp3 is not required for deubiquitylating Rpb1 since additional deletion of the protein in the *Δdst1* background did not result in an increase in polyubiquitylation of Rpb1 (Figure 40, compare lanes 2 and 8). Importantly, whereas Ubp10 and Ubp12 are not required (Figure 40, compare lanes 2 with 10 and 12), Ubp6 was identified to be responsible for deubiquitylating Rpb1 upon transcriptional stalling (Fig. 40, compare lanes 1 to 4).

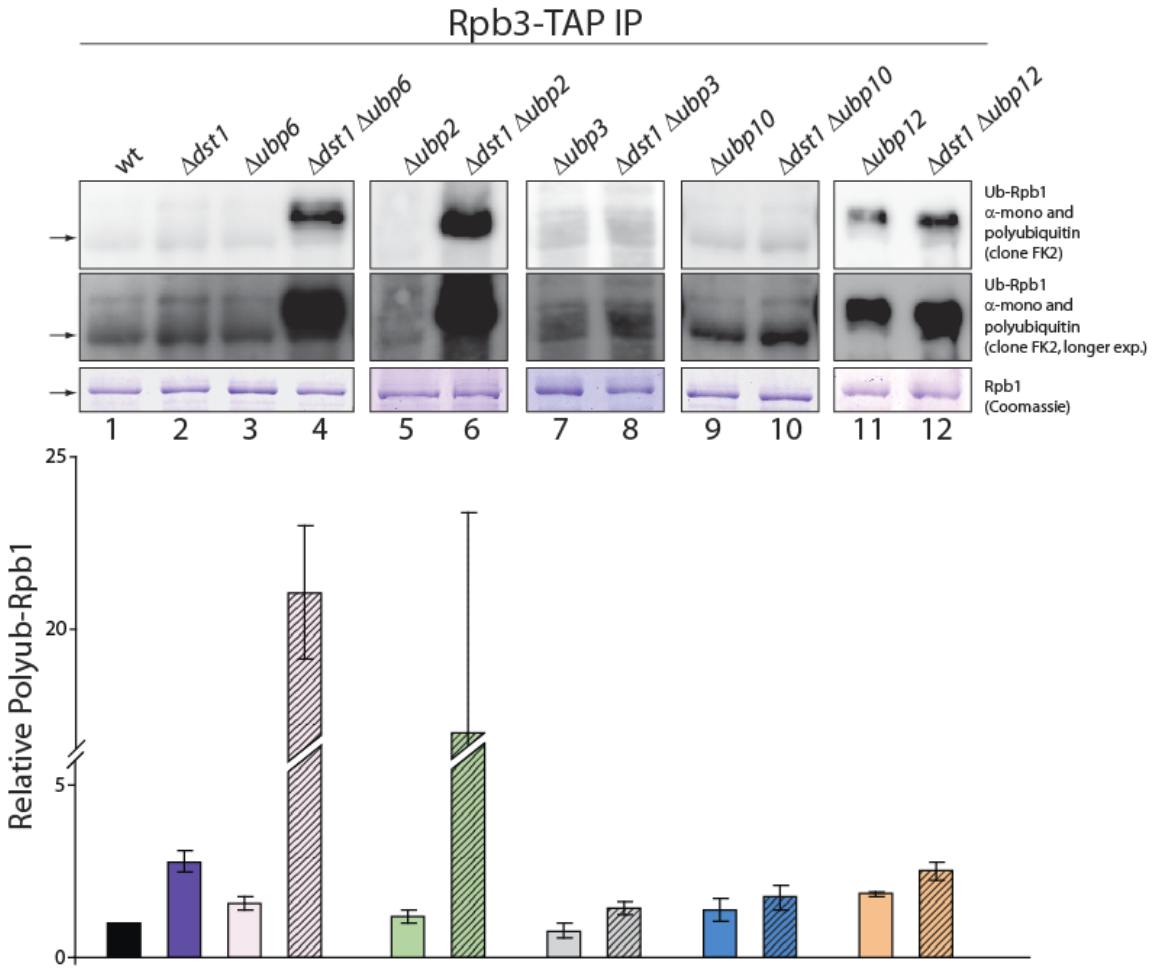


Figure 40 | Ubp2 and Ubp6 are required for deubiquitylation of transcriptionally stalled Rpb1.
RNAPII was purified from the indicated strains. Experiment as in Figure 20. Polyubiquitylation of Rpb1 is increased, *i.e.* deubiquitylation of Rpb1 is decreased in Δ*dst1* cells that also carry a deletion in the Ubp6 and Ubp2 deubiquitylases. However, there is no effect observed upon deletion of Ubp3, Ubp10 and Ubp12. Columns and error bars represent the mean ± standard deviation from 3 experiments.

Ubp6, a cysteine protease, is an abundant proteasome-associated protein, which associates with the base of the proteasome. Binding of Ubp6 to the proteasome activates Ubp6's catalytic activity (Leggett, Hanna et al. 2002), indicating an intimate functional relationship between Ubp6 and the proteasome. Recently it was reported that Ubp6 delays the breakdown of proteins by the proteasome. During this degradation delay, substrate deubiquitylation proceeds on proteasomes, but with a different mode than the one observed in the absence of Ubp6 (Hanna, Hathaway et al. 2006). Overall, it was suggested that Ubp6 has a catalytic as well as a non-catalytic function. Specifically, it deubiquitylates ubiquitylated proteins before their degradation thereby recycling ubiquitin and it delays degradation of polyubiquitylated proteins by the proteasome in a mainly non-catalytic manner (Hanna, Hathaway et al. 2006). To determine whether the catalytic activity of Ubp6 is indeed needed for deubiquitylation of Rpb1 (Figure 40) we assessed Rpb1 ubiquitylation in the catalytically inactive *ubp6-C118A* mutant that still inhibits the proteasome (Hanna, Hathaway et al. 2006). Rpb1 ubiquitylation levels are increased in *ubp6-C118A Δdst1* cells to a similar extent as in *Δubp6 Δdst1* cells showing that the deubiquitylase activity of Ubp6 is necessary for Rpb1 deubiquitylation (Figure 41).

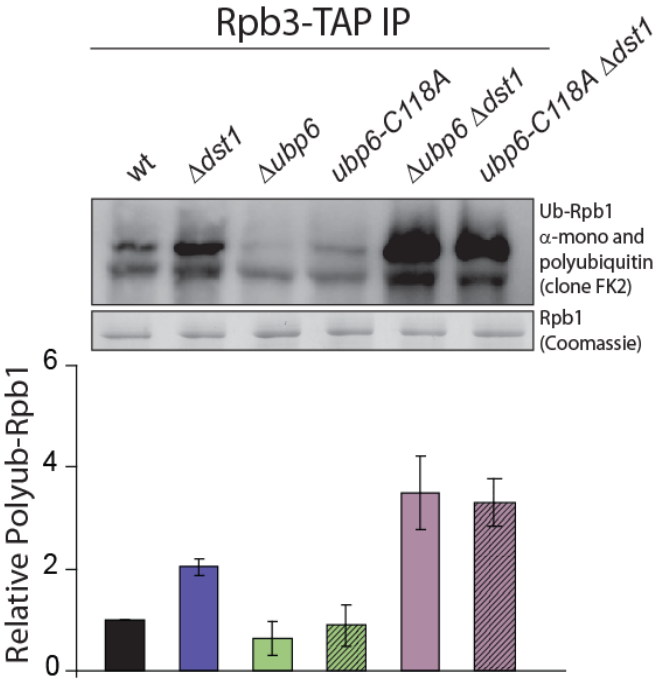


Figure 41 | The deubiquitylating activity of Ubp6 is required for Rpb1 deubiquitylation.

RNAPII was purified from the indicated strains. Experiment as in Figure 20. Polyubiquitylation of Rpb1 is increased, *i.e.* deubiquitylation of Rpb1 is decreased in *Δdst1* cells that also carry either a deletion or a catalytic-inactive mutation of Ubp6. Columns and error bars represent the mean ± standard deviation from 3 independent experiments.

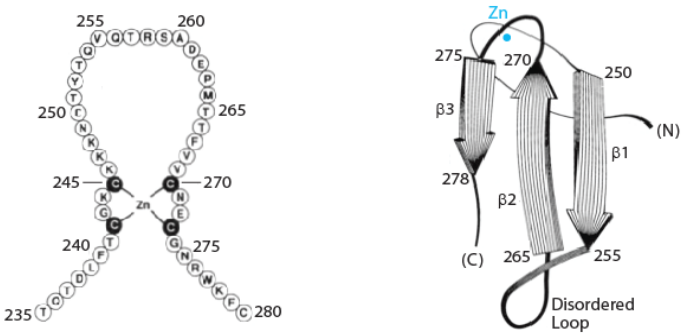
Taken together, two proteins, Ubp2 and Ubp6, are responsible for deubiquitylating Rpb1 of transcriptionally stalled RNAPII complexes.

2.8 SPECIFIC DEGRADATION UPON TRANSCRIPTIONAL IMPAIRMENT MIGHT OCCUR IN RNAPI AND RNAPIII TRANSCRIPTION.

The eukaryotic RNA polymerases (RNAPI, II and III) are the central multiprotein machines that synthesize mainly ribosomal, messenger, and transfer RNA, respectively. All three polymerases are multisubunit enzymes. Ten subunits form a structurally conserved core, and additional subunits are located on the periphery (see Introduction 1.2). Since there is a high level of similarity among the three enzymes it could be that the pathway for the controlled RNAP degradation upon block of transcription also exists for RNAPI and RNAPIII.

Several studies have shown that the transcription elongation factor Dst1 of RNAPII is homologous to the polymerase subunits Rpa12 of RNAPI and Rpc11 of RNAPIII (Prescott, Osheim et al. 2004; Huang, Intine et al. 2005). All three proteins contain a zinc-beta ribbon domain in which four cysteine residues bind to the zinc ion. This domain was shown in the case of Dst1 to be required for stimulating the RNA cleavage activity of the polymerase complex (Jeon, Yoon et al. 1994; Kettenberger, Armache et al. 2003). This domain contains 3 β-sheets and 4 highly conserved cysteine residues which are required for the binding of the zinc ion (Figure 42A).

A



B

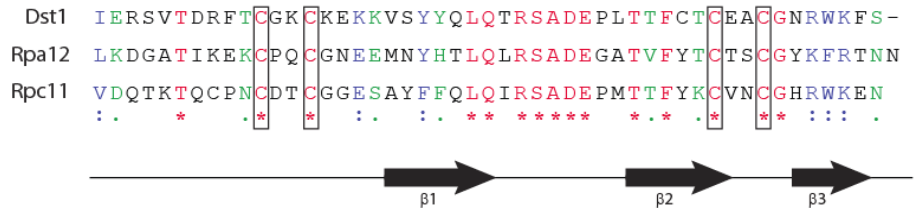


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Figure 42 | Schematic representation of the structural alignment of Dst1, Rpa12 and Rpc11.

A, Amino acid composition of the zinc-beta ribbon domain of Dst1 showing the highly conserved Cys residues that bind the zinc ion (left panel) and representation of the domain's secondary structure (right panel). The zinc ion is shown as a blue sphere. Scheme modified from (Qian, Gozani et al. 1993) **B,** Protein sequence alignment of the zinc-beta ribbon domains of the homolog proteins Dst1, Rpa12 and Rpc11. Rectangulars indicate the highly conserved Cys residues. Identical amino acids (*****) are shown in red, conservative substitutions (**:**) in blue and semi-conservative ones (**.**) in green. Alignment was done with ClustalW2.

Sequence alignment of the zinc-beta ribbon domains of the three homologous proteins – Dst1, Rpa12 and Rpc11– reveals their high level of similarity (Figure 42B).

In order to impair transcription elongation of RNAPI and III the Rpa12 and Rpc11 subunits, respectively, were mutated in the cells. In the case of RNAPI the Rpa12 subunit was deleted and the resulting strain even though viable exhibited a growth defect and both the wild-type strain and the mutant had to be grown at lower temperatures (20°C) (Figure 43A, top panel). For RNAPIII the Rpc11 subunit was mutated via random mutagenesis to produce a temperature sensitive (ts) mutant that had impaired Rpc11 function (Ben-Aroya, Coombes et al. 2008).

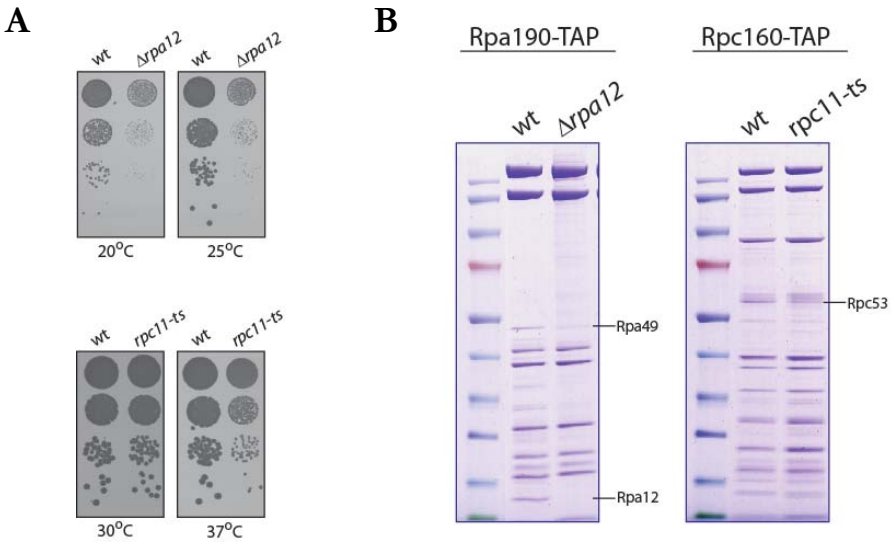

Figure 43 | Growth and subunit integrity of the "TFIIS-like" mutants of RNAPI and RNAPIII.

A, Growth analysis of the "TFIIS-like" mutants, *Δrpa12* and *rpc11-ts*. 10-fold serial dilutions of the indicated yeast strains were spotted onto YPD plates and incubated at different temperatures. **B,** RNAPI and RNAPIII

(continue from previous page)

subunit composition upon transcriptional stalling. TAP-purification of the two enzymes in the *Δrpa12* and *rpc11-ts* strains.

The *rpc11-ts* mutant exhibited a growth defect at the non-permissive temperature (37°C) (Figure 43A, bottom panel). More importantly, in these mutants the subunit composition of both RNAPI and RNAPIII is not altered indicating that the multisubunit enzymes do not fall apart upon loss-of-function of Rpa12 and Rpc11, respectively (Figure 43B).

To investigate whether RNAPI and III are degraded upon transcription elongation impairment the total cellular levels of their corresponding biggest subunits were quantified in the *Δrpa12* and *rpc11-ts* mutant strains. As shown in Figure 44, in both of the cases the deletion of the "Dst1-like" subunit resulted in a dramatic reduction in the total cellular levels of Rpa190 (for RNAPI) and of Rpc160 (for RNAPIII) as compared to wild-type cells.

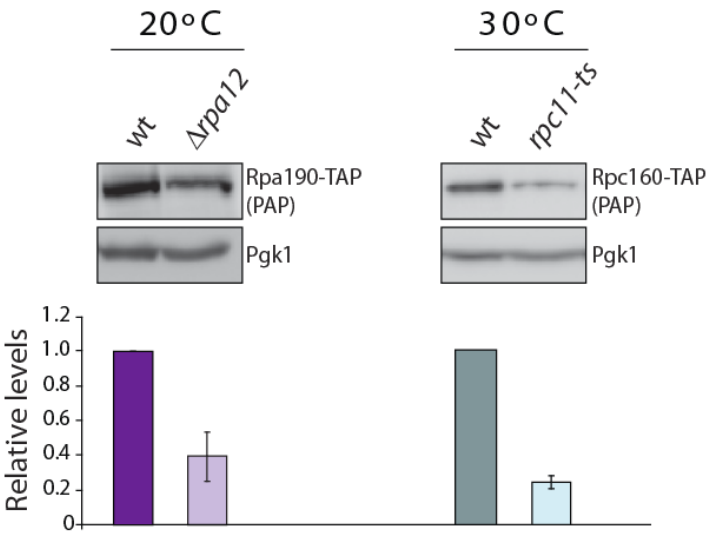

Figure 44 | Mutation of the 'Dst1-like' subunit of RNAPI and III results in the degradation of their corresponding largest subunit Rpa190 and Rpc160. Total cellular levels of Rpa190-TAP and Rpc160-TAP were assessed by western blot with the PAP antibody in the corresponding strains at the indicating temperatures. Experiment as in Figure 12. Columns and error bars represent the mean ± standard deviation from 3 independent experiments

Taken together, it seems that there is also transcriptional elongation impairment-dependent degradation of RNAPI and RNAPIII. However, it is crucial to investigate whether the *RPA190* and *RPC160* mRNA levels are affected in the mutant cells. Additionally, to address if Rpa190 and Rpc160 are targeted for UPP-mediated degradation the polyubiquitylation levels of these two proteins should be assessed. Whether the molecular mechanism for the polyubiquitylation and degradation of Rpa190 and Rpc160 is similar to the one employed in the case of Rpb1 remains to be uncovered.

3. DISCUSSION

Transcription is the first step in gene expression. By being the first it is one of the most tightly regulated processes and it requires a plethora of associating factors which facilitate the efficient progression through the transcription cycle (Saunders, Core et al. 2006). The coordinated function of the transcribing complex with the numerous transcription factors results in the accurate and fast transcription of all protein coding genes and could be resembled with the function of a intricate machine performing a delicate yet highly productive task (Panning and Taatjes 2008). However, even though transcription was once considered to be a step-by-step it is now known that the different stages of transcription are inter- and intra-connected with proteins from one phase (e.g. initiation) functioning in the other two stages (e.g. elongation or termination) and vice versa (*e.g.*, transcription elongation factors implicated in other stages of gene expression; see Introduction 1.6). Importantly, the study of transcription elongation revealed that it is also a highly dynamic and discontinuous process. It was discovered that about 4% of the polymerases that actively transcribe become arrested on the gene for times that can last up to four minutes. During transcript elongation RNAPII engages in pausing, arresting and backtracking both *in vitro* and *in vivo* (see Introduction 1.5). Given the thousands of mRNAs produced by the cell every minute an irreversible arrest of RNAPII on an essential gene could compromise cell viability. This study elucidates the molecular mechanism by which the cell copes with the irreversible arrest of the transcribing complex during transcription elongation. The newly identified pathway for the transcriptional stalling-dependent degradation of RNAPII was termed TRADE.

3.1 Transcriptional Stalling-Dependent Degradation of RNAPII-The TRADE Pathway

3.1.1 Transcriptional impairment results in lower Rpb1 levels.

In order to be able to elucidate the TRADE pathway, three different approaches were used to impair transcription elongation and thus to increase transcriptional stalling events.

These were: (i) deletion of four transcription elongation factors known to be required for efficient transcription elongation –which were used mainly throughout the study–, (ii) nucleotide depletion which mimics natural conditions of cell starvation and (iii) elevated growth temperature which mimics natural conditions of environmental changes. In all three conditions degradation of RNAPII's largest subunit Rpb1 was observed (Figures 12, 16, 18 and 19).

Moreover, the recruitment of the transcribing complex to highly transcribed genes was also reduced (Figure 11B). This finding corroborated a number of studies showing the reduced RNAPII occupancy on the genes in transcription elongation mutant cells (Hartzog, Wada et al. 1998; Keogh, Podolny et al. 2003; Xiao, Shibata et al. 2007; Ahn, Keogh et al. 2009; Sigurdsson, Dirac-Svejstrup et al. 2010) and references therein). The reduced recruitment of Rpb3, and hence of RNAPII, across the gene (Figure 11B) most likely results from the lower protein levels of Rpb1 shown in Figure 12.

Importantly, the reduction in the cellular protein levels of Rpb1 (Figure 12) were a result of the proteolytic degradation of the protein rather than of the reduction of its corresponding mRNA (Figure 13).

Taken together, this study showed that transcriptional impairment leads in the proteolytic degradation of the largest subunit of RNAPII, Rpb1.

3.1.2 Specific degradation of Rpb1 at the site of transcription probably leads to the disassembly of the stalled complex.

The transcriptionally stalled RNAPII is targeted for UPP-mediated degradation since the levels of polyubiquitylated Rpb1 are significantly increased in the four transcription elongation mutants (Figure 20). The polyubiquitylated RNAPII is probably still bound to the transcribed strand of the DNA. Degradation at the site where the RNAPII complex became stalled seems reasonable since only then the gene can be freed for transcription by subsequent polymerases. If the arrested RNAPII had the ability to go away and be released from the DNA then the mechanism elucidated in this study would not be necessary.

Consistently, RNAPII in a ternary complex with RNA and DNA is the preferred substrate for polyubiquitylation (Somesh, Reid et al. 2005).

Supporting this idea, the recruitment of the catalytic core of the 26S proteasome is significantly increased in the mutant cells. Specifically, the proteasomal occupancy was much higher towards the middle and 3' end of the ORF which most probably reflects the site of the transcriptional stalling (Figure 24). Additionally, the association of the proteasome with its substrate RNAPII is also increased (Figure 23). These two observations indicate that the proteasome is responsible for the degradation of the polyubiquitylated Rpb1 of irreversibly stalled RNAPII at the site of transcription.

One important observation is that only the levels of Rpb1 are reduced in the mutant cells while the levels of another RNAPII subunit, Rpb3, remained unaffected (Figure 12C). Consistently, upon DNA damage-induced stalling only Rpb1 is degraded while the other 11 subunits are not (Malik, Bagla et al. 2008). The specific degradation of RNAPII's largest subunit indicates that this is probably sufficient for the release of the stalled complex from the site of transcription/stalling. Most probably, once the core Rpb1 subunit is degraded then the rest of the polymerase falls apart. Furthermore, the associated transcription factors most likely also disassemble from the transcribing complex (Figure 14). Both the released RNAPII subunits and the transcription factors are probably recycled and used for another round of transcription.

3.1.3 K63-linked polyubiquitylation of Rpb1 leads to its degradation *in vivo*.

For a protein to be degraded by the proteasome a polyubiquitin chain has to be attached to it. However, addition of a short ubiquitin chain in a protein might also serve for regulatory purposes (see Introduction 1.7.1). In any case the nature of the polyubiquitin chain attached is an indication of whether the protein should be degraded or regulated. In Rpb1 of transcriptionally stalled RNAPII there are mainly K63-linked chains (Figure 30).

Until recently it was believed that only K48-linked polyubiquitin chains are targeting a protein for degradation. However, it was reported that K63-linked chains can also serve for

degradation purposes. Specifically, it was shown that polyubiquitin chains of transcriptionally stalled mammalian RNAPII formed *in vitro* are also K63-linked (Lee and Sharp 2004). Moreover, proteins carrying K63-linked polyubiquitin chains could be degraded by the proteasome both *in vitro* and *in vivo* (Kirkpatrick, Hathaway et al. 2006; Kim, Kim et al. 2007; Saeki, Kudo et al. 2009). Therefore, the observed K63-polyubiquitylated Rpb1 could lead to RNAPII degradation in the TRADE pathway. Consistent with this hypothesis, this study showed that K63-linked polyubiquitin chains are needed *in vivo* for Rpb1 degradation caused by transcriptional stalling (Figure 31). However, in the *Δctk1*, *Δdst1*, *Δtho2*, and *Δbur2* cells there is also a small increase in the K48-linked chains (Figure 30). So, it could be that there are two distinct populations of polyubiquitylated Rpb1 –one with K48- and the other K63-linked chains– inside the cell with one of them being more rapidly degraded than the other. In addition, it can not be excluded that the mainly found K63-polyubiquitin chains are remodelled by the function of deubiquitylases and K48-linked chains are added by a yet unknown E3 ligase for efficient degradation of Rpb1 (also see below).

The polyubiquitin chain is attached to an internal lysine residue of the targeted substrate. In general more than one lysine residue within a protein can serve as ubiquitylation sites. In the TRADE pathway the polyubiquitin chain is attached to K330 and K695 of Rpb1. K330 (Figure 32, magenta lysine) lies in a protein domain that is unordered in free RNAPII, but ordered in the elongating form while K695 (Figure 32, blue lysine) is located more than 125 Å away from the first one. The location of the K330 residue –structured only upon transcription elongation– allows the explanation of the preferential ubiquitylation of the elongating form of RNAPII (also see below).

Taken together, in the TRADE pathway Rpb1 of transcriptionally stalled RNAPII is polyubiquitylated on K330 and K695 via K63-linked chains which are necessary for its *in vivo* degradation.

#

3.1.4 Components for the attachment of the polyubiquitin chain on Rpb1

In general, polyubiquitylation is mediated by an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3) (see Introduction 1.7.1). Since there is only one E1 enzyme (Uba1) in *S.cerevisiae*, aim of this study was to uncover the E2 and E3 enzymes. Additionally, this study aimed to identify further components that either promote or inhibit polyubiquitylation of Rpb1. To that end a candidate approach was used. Since RNAPII degradation is reported in response to DNA damage (see Introduction 1.8) the components of this pathway were tested for a function in the TRADE pathway. Indeed, the functional overlapping proteins Ubc4 and Ubc5 are the responsible E2 conjugating enzymes in the TRADE pathway (Figure 34). Moreover, Def1 is promoting Rpb1 polyubiquitylation upon transcriptional stalling since its deletion in the *Δdst1* background resulted in a significant reduction in the levels of polyubiquitylated Rpb1 observed in the single *Δdst1* strain (Figure 38A).

Importantly and in contrast to the DNA damage dependent pathway, the E3 ligase Elc1 does not ubiquitylate Rpb1 of transcriptionally stalled RNAPII (Figure 36B). Instead, polyubiquitylation of Rpb1 occurs mainly by the E3 Rsp5 (Figure 36A). This is consistent with the finding that in the TRADE pathway there are mainly K63-linked chains (Figure 30) since Rsp5 is shown to produce K63-linked polyubiquitin chains on its substrates. (Saeki, Kudo et al. 2009). Additionally, it was shown that Rsp5-mediated K63-polyubiquitylated Rpb1 is readily degraded by the 26S proteasome *in vitro* whereas nonphysiological polyubiquitylated substrates (e.g., Rsp5 and GFP) are not (Saeki, Kudo et al. 2009). Along these lines K63-linked polyubiquitin chains result in the degradation of transcriptionally stalled Rpb1 *in vivo* (Figure 31). The involvement of Rsp5 and not Elc1 as well as the requirement of K63-linked chains for the degradation of Rpb1 further corroborate the distinction between the TRADE and the DNA damage dependent pathway.

However, since there is a small increase in K48-linked chains also observed (Figure 30) it cannot be excluded the K63-linked polyubiquitin chains are remodelled by deubiquitylases and K48-linked chains are then added by a yet unknown E3 ligase. Asr1 and Bre1 were

tested as potential novel E3 enzymes but were not found to be responsible for polyubiquitylating Rpb1 of transcriptionally stalled RNAPII (Figure 37).
Taken together, polyubiquitylation of Rpb1 of transcriptionally stalled RNAPII complexes is mediated by an overlapping but different set of enzymes than polyubiquitylation of Rpb1 in the DNA damage-dependent pathway. Importantly, this is the first evidence that the mechanism of polyubiquitylation of Rpb1 is dependent on the cause of stalling.

3.1.5 Distinction and relationship between TRADE and the DNA damage-dependent pathway

The details of the two pathways are depicted in Figure 45. As mentioned above, this study shows that the cell probably distinguishes between RNAPII complexes arrested on the gene by different causes.
However, there is also an amount of resemblance between the two pathways. The first steps of Rpb1 polyubiquitylation are mediated by Ubc4, Ubc5, and Def1 and are thus identical in both pathways. The two pathways then diverge maybe by the action of DNA repair factors that are only present in case of DNA damage dependent stalling and could *e.g.* recruit Elc1. Once RNAPII is recognized as transcriptionally stalled, ubiquitin moieties could be added by the E3 Rsp5.
The involvement of only Rsp5 and not Elc1 in the TRADE pathway is also reflected in the main presence of K63-linked polyubiquitin chains since Rsp5 is shown to produce K63-linked chains *in vivo* (Saeki, Kudo et al. 2009). In contrast to the DNA damage-dependent pathway, a slow addition of K63-linked ubiquitin moieties could be essential for the transcriptional stalling-dependent pathway, as transcriptional stalling is known to occur often. It is generally accepted that proteasomal destruction of a ubiquitylated substrate requires a chain with a length of at least four ubiquitin molecules attached (Daulny and Tansey 2009) and references therein). This threshold in the number of ubiquitins required for proteolysis sets a temporal limit for the lifetime of a polyubiquitylated substrate –from the time of addition of the first ubiquitin to at least the time of addition of the fourth. So,

it could be that Rsp5-dependent ubiquitylation of Rpb1 creates a defined time window in which transcription factors attempt to restart the transcribing complex before the arrested complex is disassembled. Slow polyubiquitylation of Rbp1 would ensure that only prolonged stalling will lead to degradation.

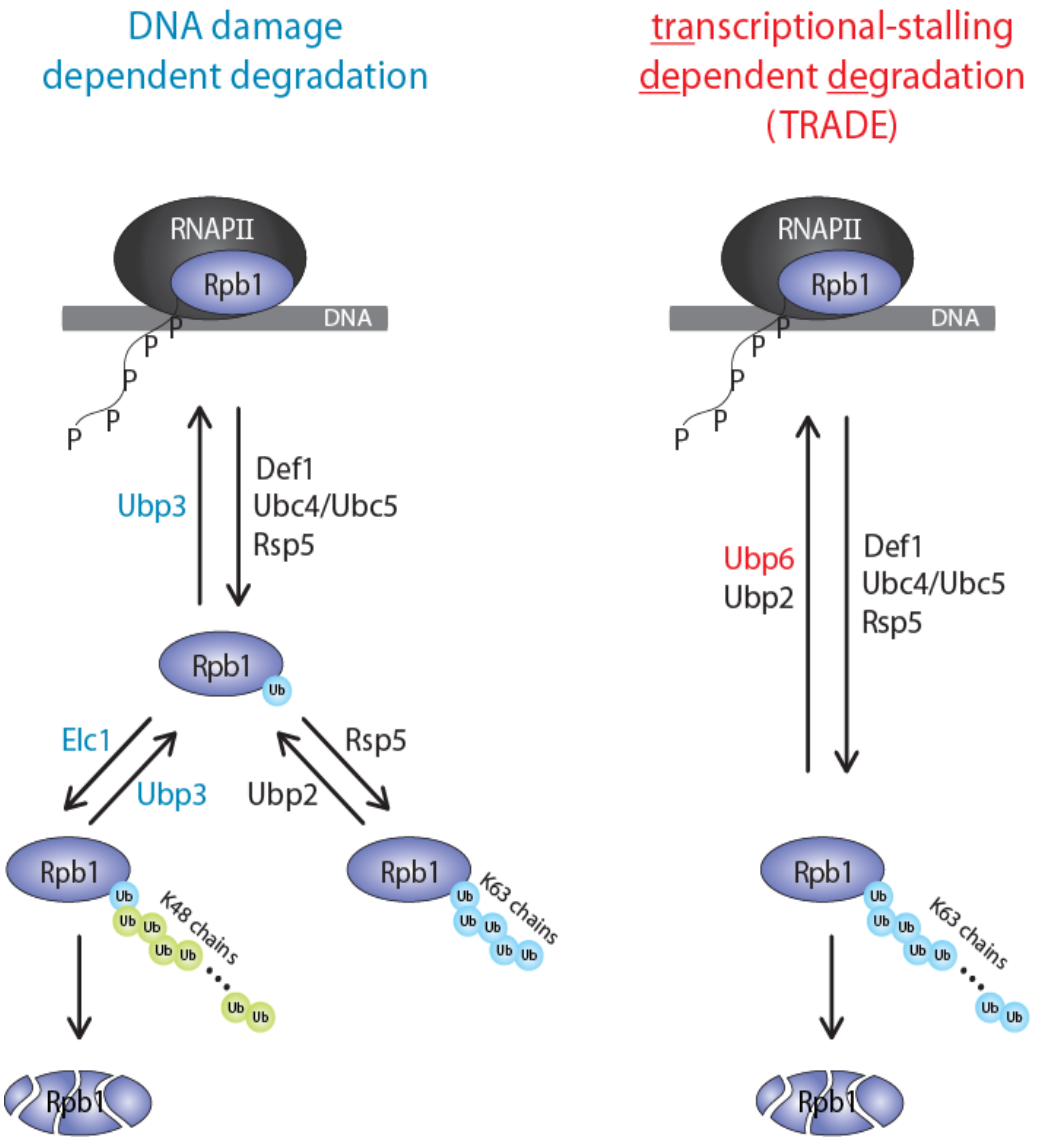

Figure 45 | Models comparing the DNA damage-dependent and the TRADE pathways.
The pathway for degradation upon DNA damage is shown on the left side and the pathway for transcriptional stalling-dependent degradation of Rpb1 is shown on the right side. See text for details.

However, one cannot exclude the presence of a second E3 ligase in the TRADE pathway. This E3 could be responsible for the slight increase in the levels of K48-linked chains observed in the four transcription elongation mutants (see Discussion 3.1.4).

Another difference between the two pathways is the enzymes responsible for deubiquitylation of Rpb1 (Figure 40). Consistent with Elc1 not involved in the TRADE pathway, Ubp3, the deubiquitylase that removes ubiquitin added by Elc1 in the DNA damage dependent pathway, is also not responsible for deubiquitylating Rpb1 of transcriptionally stalled RNAPII (Figures 36B and 40). Importantly, in the TRADE pathway an additional DUB was identified, Ubp6 (see Discussion 3.2).

Taken together there are both similarities and differences among the two pathways. Based on the data presented in this study it seems that the TRADE pathway is a distinct yet simpler version of the DNA damage dependent pathway. One hypothesis to explain this simplicity could be that the TRADE pathway was developed initially in order to cope with stalling occurring during transcription elongation and was then employed also when the transcription machinery became stalled after DNA damage. This could explain the additional enzymes involved upon DNA damage and more importantly the difference in the final "product" of the ubiquitylation reaction: K63-polyubiquitylated Rpb1 for TRADE and K48-linked chains for DNA damage. However, further analysis of the TRADE pathway might reveal a more complex pathway than the one presented here. This would indicate that the two pathways started as one but then became different to be able to distinguish the two types of stalled RNAPII. Moreover, different cellular pathways to eliminate stalled RNAPII dependent on the cause of stalling might be necessary: When a DNA damage on a transcribed gene cannot be repaired the stalled polymerase should be degraded quickly. In contrast, since transcriptional stalling occurs frequently (Darzacq, Shav-Tal et al. 2007) it is crucial that not every paused polymerase is immediately degraded. Additionally, in the two pathways the arrested transcribing complex depends on the action either of TCR factors (for DNA damage dependent) or of transcription elongation factors (for TRADE) for a "restart". So, the difference in Rpb1 deubiquitylation between the two pathways seems reasonable since the rescue-from-arrest mechanism is also different. Thus, once RNAPII is recognized as transcriptionally stalled ubiquitin moieties

could be added slowly by Rsp5 (see above). However, when RNAPII is able to continue transcription –by the function of transcription elongation factors – the polyubiquitin chain is removed by one of the two deubiquitylases, Ubp2 and Ubp6, and Rpb1 spared from degradation.

3.2 Deubiquitylation of Rpb1 opens a time window for degradation of RNAPII.

It is surprising that the proteasome interacts stably with its substrate RNAPII. As degradation of Rpb1 is potentially very deleterious, the proteasome might have to stay associated with RNAPII for some time before it can degrade Rpb1. This, along with the slow addition of the K63-linked chains (see Discussion 3.1.5), would open another time window for transcription elongation factors to rescue the stalled RNAPII complex. Along these lines, deubiquitylation of Rpb1 can also be used to control the fate of the modified protein. Ubiquitin chain "editing" and substrate deubiquitylation provide an additional level of regulation that could delay or promote the destruction of Rpb1. This fits very well to the newly identified Ubp6 being a deubiquitylase of Rpb1 to counteract degradation (Figure 40). Ubp6 is one of two proteasome-associated deubiquitylases (Hanna, Hathaway et al. 2006; Reyes-Turcu, Ventii et al. 2009) and references therein). In addition to its catalytic activity, Ubp6 delays proteasomal degradation directly by binding to the proteasome (Hanna, Hathaway et al. 2006). One function of Ubp6 is to recycle ubiquitin from polyubiquitylated substrates before their degradation (Leggett, Hanna et al. 2002). In addition, the proteasome inhibitory function of Ubp6 is thought to delay the decision to degrade a substrate (Hanna, Hathaway et al. 2006). Since Ubp6 progressively deubiquitylates the substrate protein during its delay of proteasomal degradation, the length of inhibition is crucial: When the polyubiquitin chain is shortened beyond a critical length the substrate will be released from the proteasome and thus spared from degradation (Hanna, Hathaway et al. 2006). Additionally, Ubp2 has been suggested to maintain short ubiquitin chains on Rsp5 substrates in order to spare them from degradation (Saeki, Kudo

et al. 2009). Thus, both deubiquitylases, Ubp2 and Ubp6, uncovered here to deubiquitylate Rpb1 upon transcriptional stalling could act as a "fail-safe mechanism" to spare Rpb1 from degradation, either when RNAPII resumes transcription after prolonged arrest or after erroneous ubiquitylation. This deubiquitylation of Rpb1 by Ubp2 and Ubp6 could serve two purposes: to delay RNAPII degradation until transcription factors can help the stalled complex to resume transcription by maintaining short-length polyubiquitin chain and to prevent degradation of a rescued from staling RNAPII complex by cleaving off the polyubiquitin chain from Rpb1 to produce completely deubiquitylated RNAPII.

3.3 Significance of the TRADE pathway under physiological conditions

Since RNAPII stalls frequently and for prolonged times during transcription elongation (Darzacq, Shav-Tal et al. 2007) the TRADE pathway most likely has an essential function in wild-type cells. Consistently, as shown in Figure 23, the 20S catalytic core of the proteasome stably associates with RNAPII in wild-type cells. Based on this observation one would expect that the proteasome "travels" along with RNAPII during transcription. Indeed, the proteasome is recruited to transcribed genes in wild-type cells (Gillette, Gonzalez et al. 2004; Auld, Brown et al. 2006; Sikder, Johnston et al. 2006). Importantly, the basic level of polyubiquitylated Rpb1observed in wild-type cells is reduced in cells carrying *rpb1-N488D*, an allele causing a slower polymerization rate of RNAPII that is most likely less prone to stalling (Figure 22B). Thus, less frequent stalling of RNAPII than in a wild-type situation causes less polyubiquitylation of Rpb1. This observation indicates that Rpb1 is polyubiquitylated in wild-type cells upon transcriptional stalling. This is corroborated by the recent finding that cells in which RNAPII is unable to resume from pausing also exhibit increased levels of polyubiquitylated Rpb1 (Sigurdsson, Dirac-Svejstrup et al. 2010).

In addition, Rpb1 is degraded upon treatment with 6AU, a drug that causes increased transcriptional stalling due to depletion of nucleotide pools, a situation that might mimic natural conditions such as starvation (Figure 16). Moreover, degradation of Rpb1 is also

observed upon elevated growth temperatures (Figures 18 and 19). Thus, this pathway could be essential for survival under suboptimal growth conditions.

3.4 The TRADE pathway is probably conserved in higher eukaryotes

Transcription-coupled and DNA damage-dependent ubiquitylation and degradation of RNAPII are also reported for higher eukaryotes (Lee, Wang et al. 2002). Specifically, it was shown that transcriptional arrest induced by α-amanitin, an inhibitor of RNAPII elongation, resulted in polyubiquitylation of the largest subunit of RNAPII. For ubiquitylation to take place, RNAPII had to be engaged in transcription elongation since the hyper-phosphorylated form of RNAPII was the one to be ubiquitylated and addition of template DNA promoted the reaction (Lee, Wang et al. 2002). Moreover, human cells depleted of the Rsp5-homologue NEDD4 or of the Elc1-homologue Elongin C or some Elongin C-associated proteins were compromised for RNAPII ubiquitylation/degradation (Ribar, Prakash et al. 2006; Anindya, Aygün et al. 2007; Ribar, Prakash et al. 2007). Importantly, and consistently with the TRADE pathway, NEDD4 was found to polyubiquitylate the hRpb1 *in vitro* (Beaudenon, Huacani et al. 1999) and the polyubiquitin chains formed on hRpb1 upon α-amanitin treatment were also K63-linked (Lee, Wang et al. 2002; Lee and Sharp 2004; Arima, Nitta et al. 2005).
Thus, the TRADE pathway is most likely evolutionarily conserved in higher eukaryotes.

3.5 Open questions and future directions

Following the identification of the TRADE pathway, a number of questions arise. The most important of which are mentioned below.

3.5.1 Recognition of the stalled complex

Transcriptional stalling as well as DNA damage leads to the degradation of RNAPII. How stalled RNAPII is recognized by the cell in either pathway remains an open question. However, independent of the cause of stalling the proteins XPG/Rad2 and CSB/Rad26 bind to the stalled RNAPII forming a so-called "supracomplex" (Sarker, Tsutakawa et al. 2005). Rad26 in turn forms a stable complex with the ubiquitylation promoting protein Def1 (Woudstra, Gilbert et al. 2002). *In vivo* Rad26 is unlikely to be essential for recognition of transcriptionally stalled RNAPII as deletion of *RAD26* does not affect polyubiquitylation of Rpb1 (Figure 38B). In contrast, Def1 could be involved in recognition of stalled RNAPII since it promotes ubiquitylation of Rpb1 in both pathways (Figure 38A and (Reid and Svejstrup 2004). Alternatively, a DNA-binding protein sliding on the DNA could be responsible for the identification of stalled transcribing complexes on the genes. As soon as this protein comes across an arrested RNAPII it could alert the cellular mechanism for its removal.

3.5.2 Hints for the import/export of RNAPII

It is still unknown how the RNAPII enzyme is assembled after translation of its individual subunits. Two possibilities exist: either RNAPII assembles in the cytoplasm –after translation of the 12 subunits– and is then imported to the nucleus through the nuclear pore complex (NPC) as a multi-protein complex (Figure 46A) or the individual subunits are being imported separately and then RNAPII assembles inside the nucleus (Figure 46B). However, since none of the subunits contain a nuclear localization signal (NLS) (Cramer, Bushnell et al. 2001) the assembly of RNAPII in the cytoplasm (Figure 46A) seems more likely to be true.

This study shows that there is specific degradation of Rpb1 –and not of the other subunits- inside the nucleus. This would mean that the rest of the subunits are either exported from the nucleus and then reassembled as a functional RNAPII in the cytoplasm (Figure 46A) or

that they reassemble in the nucleus using Rpb1 from the pool already present there (Figure 46B). Taken together, it would be interesting to identify which of these two pathways for the nuclear import of RNAPII is true.

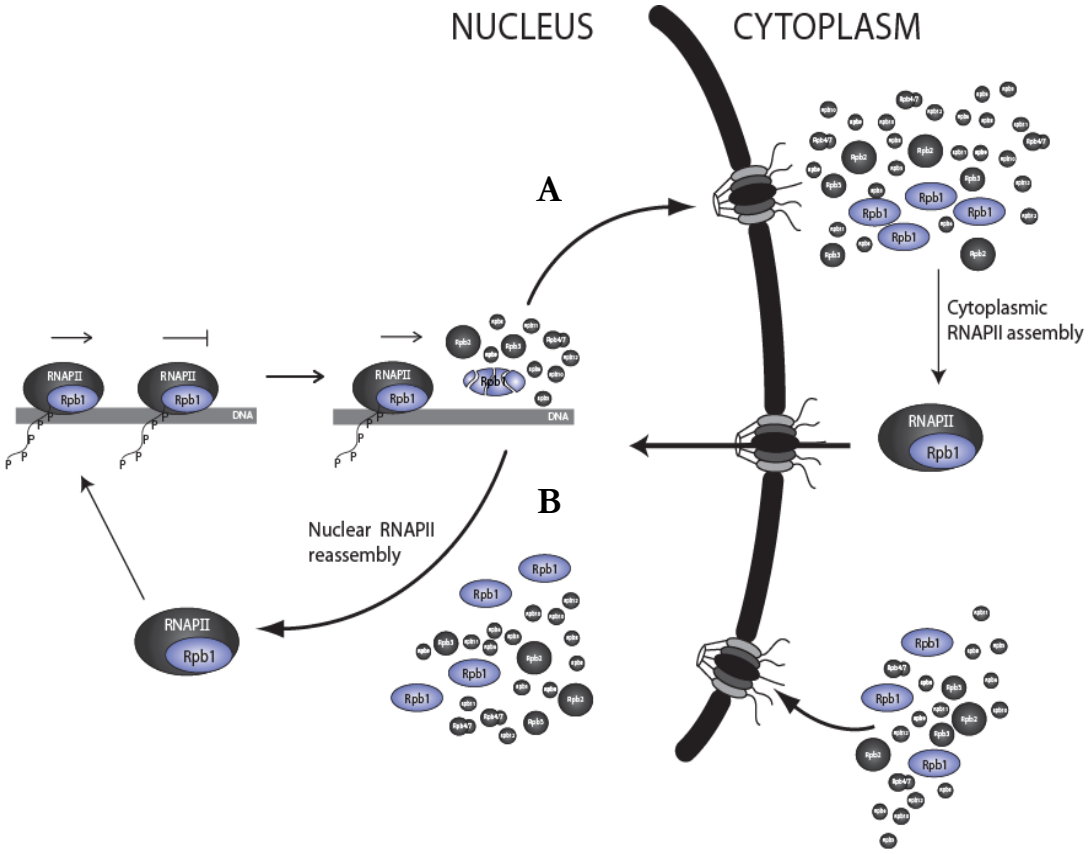

Figure 46 | Schematic representation of the two possible ways for the nuclear import of RNAPII.
A, Cytoplasmic RNAPII assembly and import of the assembled RNAPII complex. **B,** Import of individual RNAPII subunits and nuclear RNAPII assembly. See text for details.

3.5.3 Molecular mechanism for the removal of transcriptionally stalled RNAPI and RNAPIII

In this study, it is reported for the first time that inhibition of transcript cleavage by RNAPI and RNAPIII results in reduced protein levels of their corresponding largest subunit (Figure 44). It would be of great interest to investigate whether the observed reduction is a result of the UPP-mediated degradation of these proteins. If this is the case, it would be interesting to elucidate the molecular mechanism behind the ubiquitylation and degradation of Rpa190 and Rpc160 upon transcription elongation impairment of RNAPI and RNAPIII, respectively.

3.6 Model of the TRADE Pathway

This study demonstrates the existence of a novel pathway for transcriptional stalling-dependent degradation of RNAPII termed TRADE. A model for the TRADE pathway could be formulated as follows (Figure 47).

Transcription elongation is a tightly regulated and discontinuous process including frequent pauses of RNAPII. Paused RNAPII complexes resume transcription with the help of transcription elongation factors (Saunders, Core et al. 2006). In the case that transcription elongation factors fail to restart RNAPII, the persistently stalled RNAPII complex prevents transcription and has to be recognized and degraded by the cell. The first step for this removal is mediated by the ubiquitylation promoting protein Def1, the E2 conjugating enzymes Ubc4/Ubc5 and the E3 ligase Rsp5.

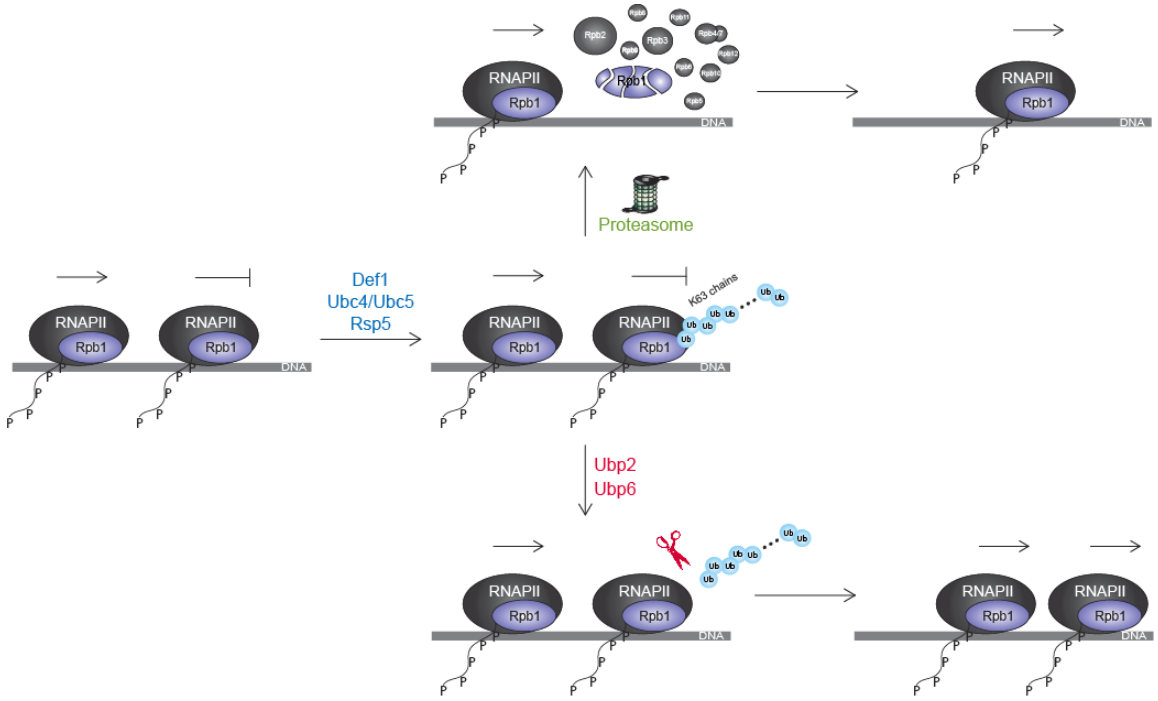

Figure 47 | Model of the TRADE pathway.

Persistently stalled RNAPII complexes are polyubiquitylated with K63-specific polyubiquitin chains by the E2s Ubc4 and Ubc5, the E3 Rsp5, and Def1. When transcription factors restart RNAPII, Rpb1 is deubiquitylated by Ubp2 and Ubp6 (lower panel) and RNAPII resumes transcription. Alternatively, as a failsafe mechanism when RNAPII remains persistently stalled, polyubiquitylated Rpb1 is recognized by the proteasome and degraded (upper panel) freeing the way for following RNAPII complexes.

The function of these proteins results in the attachment of a K63-linked polyubiquitin chain to the largest subunit of RNAPII, Rpb1. Then, the polyubiquitylated Rpb1 is degraded by the proteasome. The specific degradation of Rpb1 probably results in the disassembly of RNAPII from the site of stalling. This disassembly frees the gene and allows subsequent polymerases to efficiently transcribe the gene (Figure 47, upper panel).

Since transcriptional stalling occurs frequently (Darzacq, Shav-Tal et al. 2007) it is crucial that not every paused polymerase is immediately degraded. Two time windows ensure that RNAPII degradation only takes place when it is absolutely necessary (see Discussion 3.1.5. and 3.2). The first one is the slow addition of K63-linked polyubiquitin chains on Rpb1 by the E3 ligase Rsp5. The second one is the trimming of the expanding chains by Ubp2 and the proteasome associated Ubp6 to maintain short ubiquitin chains that do not trigger proteasomal degradation. When transcription elongation factors succeed in "restarting" the stalled RNAPII within this time the polyubiquitin chain is completely removed by one of the two deubiquitylases, Ubp2 and Ubp6, and Rpb1 spared from degradation (Figure 47, lower panel). The "rescued" RNAPII is then able to resume transcription.

In summary, the TRADE pathway elucidated in this study ensures continued transcription.

4. MATERIALS

4.1. STRAINS

4.1.1. *Escherichia coli* strains

Table 3 | *Esherichia coli* strains used in this study.

Name	Genotype	Source
DH5α F-	ϕ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1,*hsdR*17(rk-, mk+), *phoA*, *supE*44, λ-, *thi*-1, *gyrA*96, *relA*1	Stratagene
BL21 DE3 F–	ompT; hsdS(rB, mB); dcm+; Tetr; gal λ(DE3) endA; Hte [argU, ileY, leuW, Camr]	Stratagene

4.1.2. *Saccharomyces cerevisiae* strains

Table 4 | *Saccharomyces cerevisiae* strains used in this study.

Nr.	Name	Parent	Relevant Genotype	Reference
wild-type strains				
YKS1	RS453 (Mat a)		*ura3-1 trp1-1 his3-11,15 leu2-3,112 ade2-1 can1-100 GAL+*	R. Serrano
YKS2	RS453 (Mat alpha)		*ura3-1 trp1-1 his3-11,15 leu2-3,112 ade2-1 can1-100 GAL+*	R. Serrano
YKS8	W303 (Mat a)		*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]*	(Wallis, Chrebet et al. 1989)
YKS184	W303 (Mat alpha)		*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 [phi+]*	(Wallis, Chrebet et al. 1989)
YKS23	BY4743 (Mat a)		*his3Δ1 leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0*	(Brachmann, Davies et al. 1998)
YKS24	BY4743 (Mat alpha)		*his3Δ1 leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0*	(Brachmann, Davies et al. 1998)
YKS265	S288C (Mat alpha)		*can1Δ::MFA1pr-HIS3 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+*	(Mortimer and Johnston 1986)
YKS266	S288C (Mat a)		*can1Δ::MFA1pr-HIS3 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2+*	(Mortimer and Johnston 1986)
TAP-tagged strains				
YKS1619	RPB1-TAP	W303	*RPB1::TAP::TRP1*	This study
YKS1906	RPB2-TAP	W303	*RPB2::TAP::TRP1*	This study
YKS1138	RPB3-TAP	W303	*RPB3::TAP::TRP1*	This study
YKS1143	RPB3-TAP *Δpdr5*	W303	*PDR5::HIS3 RPB3::TAP::TRP1*	This study
YKS981	RPB3-TAP *CTK1*	W303	*CTK1::HIS3, RPB3::TAP::TRP1*	This study

	shuffle		pRS316-CTK1[URA3 CTK1]	
YKS1139	RPB3-TAP *Δpdr5 CTK1* shuffle	W303	*PDR5::HIS3 CTK1::HIS3, RPB3::TAP::TRP1* pRS316-CTK1[URA3 CTK1]	This study
YKS1030	RPB3-TAP *DST1* shuffle	W303	*DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1140	RPB3-TAP *Δpdr5 DST1* shuffle	W303	*PDR5::HIS3 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1109	RPB3-TAP *THO2* shuffle	W303	*THO2::KANMX6, RPB3::TAP::TRP1* pRS316-THO2[URA3 THO2]	This study
YKS1141	RPB3-TAP *Δpdr5 THO2* shuffle	W303	*PDR5::HIS3 THO2::KANMX6, RPB3::TAP::TRP1* pRS316-THO2[URA3 THO2]	This study
YKS1103	RPB3-TAP *BUR2* shuffle	W303	*BUR2::KANMX6, RPB3::TAP::TRP1* pRS316-BUR2[URA3 BUR2]	This study
YKS1142	RPB3-TAP *Δpdr5 BUR2* shuffle	W303	*PDR5::HIS3 BUR2::KANMX6, RPB3::TAP::TRP1* pRS316-BUR2[URA3 BUR2]	This study
YKS1649	RPB3-TAP *Δubc4*	W303	*UBC4::HIS3 RPB3::TAP::TRP1*	This study
YKS1650	RPB3-TAP *Δubc4 DST1* shuffle	W303	*UBC4::HIS3 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1652	RPB3-TAP *Δubc5*	W303	*UBC5::HIS3 RPB3::TAP::TRP1*	This study
YKS1653	RPB3-TAP *Δubc5 DST1* shuffle	W303	*UBC5::HIS3 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1654	RPB3-TAP *RSP5* shuffle	W303	*RSP5::HIS3 RPB3::TAP::TRP1*	This study
YKS1655	RPB3-TAP *Δdst1 RSP5* shuffle	W303	*RSP5::HIS3 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1656	RPB3-TAP *Δdef1*	W303	*DEF1::KANMX6 RPB3::TAP::TRP1*	This study
YKS1657	RPB3-TAP *Δdef1 DST1* shuffle	W303	*DEF1::KANMX6 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1745	RPB3-TAP *Δelc1*	W303	*ELC1::KANMX6 RPB3::TAP::TRP1*	This study
YKS1747	RPB3-TAP *Δelc1 DST1* shuffle	W303	*ELC1::KANMX6 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1834	RPB3-TAP *Δasr1*	W303	*ASR1::KANMX6 RPB3::TAP::TRP1*	This study
YKS1835	RPB3-TAP *Δasr1 DST1* shuffle	W303	*ASR1::KANMX6 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1837	RPB3-TAP *Δbre1*	W303	*BRE1::KANMX6 RPB3::TAP::TRP1*	This study
YKS1838	RPB3-TAP *Δbre1 DST1* shuffle	W303	*BRE1::KANMX6 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1840	RPB3-TAP *Δrad26*	W303	*RAD26::KANMX6*	This study

			RPB3::TAP::TRP1	
YKS1841	RPB3-TAP *Δrad26 DST1* shuffle	W303	*RAD26::KANMX6 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1746	RPB3-TAP *Δubp2*	W303	*UBP2::KANMX6 RPB3::TAP::TRP1*	This study
YKS1751	RPB3-TAP *Δubp2 DST1* shuffle	W303	*UBP2::KANMX6 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1665	RPB3-TAP *Δubp3*	W303	*UBP3::HIS3 RPB3::TAP::TRP1*	This study
YKS1666	RPB3-TAP *Δubp3 DST1* shuffle	W303	*UBP3::HIS3 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1695	RPB3-TAP *Δubp4*	W303	*UBP4::KANMX6 RPB3::TAP::TRP1*	This study
YKS1712	RPB3-TAP *Δubp4 DST1* shuffle	W303	*UBP4::KANMX6 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1696	RPB3-TAP *Δubp6*	W303	*UBP6::KANMX6 RPB3::TAP::TRP1*	This study
YKS1714	RPB3-TAP *Δubp6 DST1* shuffle	W303	*UBP6::KANMX6 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1697	RPB3-TAP *Δubp10*	W303	*UBP10::KANMX6 RPB3::TAP::TRP1*	This study
YKS1716	RPB3-TAP *Δubp10 DST1* shuffle	W303	*UBP10::KANMX6 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1698	RPB3-TAP *Δubp12*	W303	*UBP12::KANMX6 RPB3::TAP::TRP1*	This study
YKS1718	RPB3-TAP *Δubp12 DST1* shuffle	W303	*UBP12::KANMX6 DST1::HIS3, RPB3::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS982	PRE1-TAP *CTK1* shuffle	W303	*CTK1::HIS3, PRE1::TAP::TRP1* pRS316-CTK1[URA3 CTK1]	This study
YKS1042	PRE2-TAP *CTK1* shuffle	W303	*CTK1::HIS3, PRE2::TAP::TRP1* pRS316-CTK1[URA3 CTK1]	This study
YKS983	PRE4-TAP *CTK1* shuffle	W303	*CTK1::HIS3, PRE4::TAP::TRP1* pRS316-CTK1[URA3 CTK1]	This study
YKS1041	PUP1-TAP *CTK1* shuffle	W303	*CTK1::HIS3, PUP1::TAP::TRP1* pRS316-CTK1[URA3 CTK1]	This study
YKS1169	SUG1-TAP *CTK1* shuffle	W303	*CTK1::HIS3, SUG1::TAP::TRP1* pRS316-CTK1[URA3 CTK1]	This study
YKS1167	SUG2-TAP *CTK1* shuffle	W303	*CTK1::HIS3, SUG2::TAP::TRP1* pRS316-CTK1[URA3 CTK1]	This study
YKS1168	CIM5-TAP *CTK1* shuffle	W303	*CTK1::HIS3, CIM5::TAP::TRP1* pRS316-CTK1[URA3 CTK1]	This study
YKS1033	PRE1-TAP *DST1* shuffle	W303	*DST1::HIS3, PRE1::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1034	PRE2-TAP *DST1* shuffle	W303	*DST1::HIS3, PRE2::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1035	PRE4-TAP *DST1* shuffle	W303	*DST1::HIS3, PRE4::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1031	PUP1-TAP *DST1*	W303	*DST1::HIS3, PUP1::TAP::TRP1*	This study

	shuffle		pRS316-DST1[URA3 DST1]	
YKS1172	SUG1-TAP *DST1* shuffle	W303	*DST1::HIS3, SUG1::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1170	SUG2-TAP *DST1* shuffle	W303	*DST1::HIS3, SUG2::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1171	CIM5-TAP *DST1* shuffle	W303	*DST1::HIS3, CIM5::TAP::TRP1* pRS316-DST1[URA3 DST1]	This study
YKS1110	PRE1-TAP *THO2* shuffle	W303	*THO2::KANMX6, PRE1::TAP::TRP1* pRS316-THO2[URA3 THO2]	This study
YKS1111	PRE2-TAP *THO2* shuffle	W303	*THO2::KANMX6, PRE2::TAP::TRP1* pRS316-THO2[URA3 THO2]	This study
YKS1112	PRE4-TAP *THO2* shuffle	W303	*THO2::KANMX6, PRE4::TAP::TRP1* pRS316-THO2[URA3 THO2]	This study
YKS1113	PUP1-TAP *THO2* shuffle	W303	*THO2::KANMX6, PUP1::TAP::TRP1* pRS316-THO2[URA3 THO2]	This study
YKS1176	SUG1-TAP *THO2* shuffle	W303	*THO2::KANMX6, SUG1::TAP::TRP1* pRS316-THO2[URA3 THO2]	This study
YKS1175	SUG2-TAP *THO2* shuffle	W303	*THO2::KANMX6, SUG2::TAP::TRP1* pRS316-THO2[URA3 THO2]	This study
YKS1174	CIM5-TAP *THO2* shuffle	W303	*THO2::KANMX6, CIM5::TAP::TRP1* pRS316-THO2[URA3 THO2]	This study
YKS1104	PRE1-TAP *BUR2* shuffle	W303	*BUR2::KANMX6, PRE1::TAP::TRP1* pRS316-BUR2[URA3 BUR2]	This study
YKS1105	PRE2-TAP *BUR2* shuffle	W303	*BUR2::KANMX6, PRE2::TAP::TRP1* pRS316-BUR2[URA3 BUR2]	This study
YKS1106	PRE4-TAP *BUR2* shuffle	W303	*BUR2::KANMX6, PRE4::TAP::TRP1* pRS316-BUR2[URA3 BUR2]	This study
YKS1107	PUP1-TAP *BUR2* shuffle	W303	*BUR2::KANMX6, PUP1::TAP::TRP1* pRS316-BUR2[URA3 BUR2]	This study
YKS1164	SUG1-TAP *BUR2* shuffle	W303	*BUR2::KANMX6, SUG1::TAP::TRP1* pRS316-BUR2[URA3 BUR2]	This study
YKS1166	CIM5-TAP *BUR2* shuffle	W303	*BUR2::KANMX6, CIM5::TAP::TRP1* pRS316-BUR2[URA3 BUR2]	This study
YKS1201	RPB3-TAP *RPB1* shuffle	W303	*RPB1::KANMX6 RPB3::TAP::TRP1* pRP112 [URA3 RPB1]	This study
YKS1202	RPB3-TAP *RPB1* shuffle *CTK1* shuffle	W303	*RPB1::KANMX6 CTK1::HIS3 RPB3::TAP::TRP1* pRP112 [URA3 RPB1] pRS316-CTK1 [URA3 CTK1]	This study
YKS1203	RPB3-TAP *RPB1* shuffle	W303	*RPB1::KANMX6 DST1::HIS3 RPB3::TAP::TRP1*	This study

	DST1 shuffle		pRP112 [URA3 RPB1] pRS316-DST1 [URA3 DST1]	
YKS1204	RPB3-TAP *RPB1* shuffle *THO2* shuffle	W303	*RPB1::KANMX6 THO2::KANMX6 RPB3::TAP::TRP1* pRP112 [URA3 RPB1] pRS316-THO2 [URA3 THO2]	This study
YKS1205	RPB3-TAP *RPB1* shuffle *BUR2* shuffle	W303	*RPB1::KANMX6 BUR2::KANMX6 RPB3::TAP::TRP1* pRP112 [URA3 RPB1] pRS316-BUR2 [URA3 BUR2]	This study
YKS322	CTK1-TAP	RS453	CTK1::TAP::URA3	(Rother and Strasser 2007)
YKS433	DST1-TAP	RS453	DST1::TAP::TRP1	K.Sträßer (unpublished work)
YKS45	THO2-TAP	RS453	THO2::TAP::TRP1	K.Sträßer (unpublished work)
YKS238	BUR2-TAP	RS453	BUR2::TAP::TRP1	K.Sträßer (unpublished work)
YKS551	pGAL1::CTK1-TAP	RS453	HIS3::PGAL1::CTK1::TAP::URA3	(Rother and Strasser 2007)
YKS1616	pGAL1::DST1-TAP	RS453	KANMX6::PGAL1::DST1::TAP::TRP1	This study
YKS1617	pGAL1::THO2-TAP	RS453	HIS3::PGAL1::THO2::TAP::TRP1	This study
YKS1618	pGAL1::BUR2-TAP	RS453	HIS3::PGAL1::BUR2::TAP::TRP1	This study
YKS1642	RPC160-TAP	S288C	RPC160::TAP::KANMX6	This study
YKS1644	RPC160-TAP *rpc11-4*	S288C	*rpc11-4* RPC160::TAP::KANMX6	This study
YKS1662	RPA190-TAP		*RPA190::TAP::TRP1*	This study
YKS1663	RPA190-TAP *Δrpa12*		*RPA12::LEU2 RPA190::TAP::TRP1*	This study
Single mutation or deletion strains				
YKS814	*CTK1* shuffle	W303	*CTK1::HIS3* pRS316-CTK1 [URA3 CTK1]	(Rother and Strasser 2007)
YKS1027	*DST1* shuffle	W303	*DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1108	*THO2* shuffle	W303	*THO2::KANMX6* pRS316-THO2 [URA3 THO2]	This study
YKS1101	*BUR2* shuffle	W303	*BUR2::KANMX6* pRS316-BUR2 [URA3 BUR2]	This study
YKS1076	*RPB1* shuffle	W303	*RPB1::KANMX6* pRS316-RPB1 [URA3 RPB1]	This study
YKS1188	*UBC4* shuffle	W303	*UBC4::HIS3* pRS316-UBC4 [URA3 UBC4]	This study
YKS1186	*UBC5* shuffle	W303	*UBC5::HIS3* pRS316-UBC5 [URA3 UBC5]	This study
YKS1191	*RSP5* shuffle	W303	*RSP5::HIS3* pRS316-RSP5 [URA3 RSP5]	This study
YKS1425	*rsp5-2*	DF5	*RSP5::HIS3* ura3-52::rsp5-2::URA3	Gift from S.Jentsch
YKS1603	*Δubp3*	W303	*UBP3::HIS3*	This study

YKS1601	*Δubp3*	W303	*UBP3::KANMX6*	This study
YKS1743	*Δubp2*	W303	*UBP2::KANMX6*	This study
YKS1687	*Δubp4*	W303	*UBP4::KANMX6*	This study
YKS1689	*Δubp6*	W303	*UBP6::KANMX6*	This study
YKS1691	*Δubp10*	W303	*UBP10::KANMX6*	This study
YKS1693	*Δubp12*	W303	*UBP12::KANMX6*	This study
YKS1741	*Δelc1*	W303	*ELC1::KANMX6*	This study
YKS1813	*Δasr1*	W303	*ASR1::KANMX6*	This study
YKS1815	*Δbre1*	W303	*BRE1::KANMX6*	This study
YKS1817	*Δrad26*	W303	*RAD26::KANMX6*	This study
YKS1118	*Δpdr5*	W303	*PDR5::HIS3*	This study
YKS655	*cim3-1*	S288C	*cim3-1*	Gift from S.Jentsch
YKS656	*cim5-1*	S288C	*cim5-1*	Gift from S.Jentsch
YKS653	*PRE1* shuffle	DF5	*PRE1::TRP1* pDP83-PRE1 [URA3 PRE1]	Gift from S.Jentsch
YKS654	*pre1-1*	DF5	*PRE1::TRP1* pDP83-pre1-1 [URA3 pre1-1]	Gift from S.Jentsch
YKS1426	*DEF1* shuffle	W303	*DEF1::KANMX6* pRS316-DEF1 [URA3 DEF1]	This study
YKS1470	GRY3020		*RPB1*	(Malagon, Kireeva et al. 2006)
YKS1471	GRY3027		*rpb1-N488D*	(Malagon, Kireeva et al. 2006)
YKS1472	GRY3028		*rpb1-E1103G*	(Malagon, Kireeva et al. 2006)
YKS1466	Z24		*rpb2Δ297::HIS3* pRP214 [URA3 RPB2]	(Scafe, Martin et al. 1990)
YKS1467	Z422		*rpb2Δ297::HIS3* pRP2-4L [URA3 *rpb2-4*]	(Scafe, Martin et al. 1990)
YKS1468	Z425		*rpb2Δ297::HIS3* pRP2-7L [URA3 *rpb2-7*]	(Scafe, Martin et al. 1990)
YKS1469	Z428		*rpb2Δ297::HIS3* pRP2-10L [URA3 *rpb2-10*]	(Scafe, Martin et al. 1990)
YKS1639	*rpc11-4* (YDR045C/ts241-4)		*rpc11-4*	(Ben-Aroya, Coombes et al. 2008)
YKS1660	NOY504		*RPA12::LEU2*	(Nogi, Yano et al. 1993)
YKS1661	NOY505		*RPA12*	(Nogi, Yano et al. 1993)
Double mutation or deletion strains				
YKS986	*PRE1* shuffle *Δctk1*	DF5/ W303	*PRE1::TRP1 CTK1:: HIS3* pDP83-PRE1 [URA3 PRE1]	This study
YKS1193	*PRE1* shuffle *Δdst1*	DF5/ W303	*PRE1::TRP1 DST1:: HIS3* pDP83-PRE1 [URA3 PRE1]	This study

YKS1195	*PRE1* shuffle *Δtho2*	DF5/ W303	*PRE1::TRP1 THO2::KANMX6* pDP83-PRE1 [URA3 PRE1]	This study
YKS1197	*PRE1* shuffle *Δbur2*	DF5/ W303	*PRE1::TRP1 BUR2::KANMX6* pDP83-PRE1 [URA3 PRE1]	This study
YKS1120	*Δpdr5 CTK1* shuffle	W303	*PDR5::HIS3 CTK1::HIS3* pRS316-CTK1 [URA3 CTK1]	This study
YKS1128	*Δpdr5 DST1* shuffle	W303	*PDR5::HIS3 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1126	*Δpdr5 THO2* shuffle	W303	*PDR5::HIS3 THO2::KANMX6* pRS316-THO2 [URA3 THO2]	This study
YKS1124	*Δpdr5 BUR2* shuffle	W303	*PDR5::HIS3 BUR2::KANMX6* pRS316-BUR2 [URA3 BUR2]	This study
YKS1130	*CTK1* shuffle *cim3-1*	S288C /W303	*cim3-1 CTK1::HIS3* pRS316-CTK1 [URA3 CTK1]	This study
YKS1122	*CTK1* shuffle *cim5-1*	S288C /W303	*cim5-1 CTK1::HIS3* pRS316-CTK1 [URA3 CTK1]	This study
YKS1390	*DST1* shuffle *cim3-1*	S288C /W303	*cim3-1 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1392	*DST1* shuffle *cim5-1*	S288C /W303	*cim5-1 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1382	*THO2* shuffle *cim3-1*	S288C /W303	*cim3-1 THO2::KANMX6* pRS316-THO2 [URA3 THO2]	This study
YKS1384	*THO2* shuffle *cim5-1*	S288C /W303	*cim5-1 THO2::KANMX6* pRS316-THO2 [URA3 THO2]	This study
YKS1386	*BUR2* shuffle *cim3-1*	S288C /W303	*cim3-1 BUR2::KANMX6* pRS316-BUR2 [URA3 BUR2]	This study
YKS1388	*BUR2* shuffle *cim5-1*	S288C /W303	*cim5-1 BUR2::KANMX6* pRS316-BUR2 [URA3 BUR2]	This study
YKS1199	*RPB1* shuffle *CTK1* shuffle	W303	*RPB1::KANMX6 CTK1::HIS3* pRP112 [URA3 RPB1] pRS316-CTK1 [URA3 CTK1]	This study
YKS1200	*RPB1* shuffle *DST1* shuffle	W303	*RPB1::KANMX6 DST1::HIS3* pRP112 [URA3 RPB1] pRS316-DST1 [URA3 DST1]	This study
YKS1146	*RPB1* shuffle *THO2* shuffle	W303	*RPB1::KANMX6 THO2::KANMX6* pRP112 [URA3 RPB1] pRS316-THO2 [URA3 THO2]	This study
YKS1144	*RPB1* shuffle *BUR2* shuffle	W303	*RPB1::KANMX6 BUR2::KANMX6* pRP112 [URA3 RPB1] pRS316-BUR2 [URA3 BUR2]	This study
YKS1402	*Δubc4 CTK1* shuffle	W303	*UBC4::HIS3 CTK1::HIS3* pRS316-CTK1 [URA3 CTK1]	This study
YKS1404	*Δubc4 DST1* shuffle	W303	*UBC4::HIS3 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1406	*Δubc4 THO2* shuffle	W303	*UBC4::HIS3 THO2::KANMX6* pRS316-THO2 [URA3 THO2]	This study
YKS1408	*Δubc4 BUR2* shuffle	W303	*UBC4::HIS3 BUR2::KANMX6* pRS316-BUR2 [URA3 BUR2]	This study
YKS1410	*Δubc5 CTK1* shuffle	W303	*UBC5::HIS3 CTK1::HIS3* pRS316-CTK1 [URA3 CTK1]	This study
YKS1412	*Δubc5 DST1* shuffle	W303	*UBC5::HIS3 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1414	*Δubc5 THO2* shuffle	W303	*UBC5::HIS3 THO2::KANMX6* pRS316-THO2 [URA3 THO2]	This study

YKS1416	*Δubc5 BUR2* shuffle	W303	*UBC5::HIS3 BUR2::KANMX6* pRS316-BUR2 [URA3 BUR2]	This study
YKS1424	*Δubc4 Δubc5*	DF5	*UBC4::TRP1 UBC5::LEU2*	Gift from S. Jentsch
YKS1398	*Δubc4 Δubc5 CTK1* shuffle	DF5/ W303	*UBC4::TRP1 UBC5::LEU2 CTK1::HIS3* pRS316-CTK1 [URA3 CTK1]	This study
YKS1399	*Δubc4 Δubc5 DST1* shuffle	DF5/ W303	*UBC4::TRP1 UBC5::LEU2 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1400	*Δubc4 Δubc5 THO2* shuffle	DF5/ W303	*UBC4::TRP1 UBC5::LEU2 THO2::KANMX6* pRS316-THO2 [URA3 THO2]	This study
YKS1419	*RSP5* shuffle *CTK1* shuffle	W303	*RSP5::HIS3 CTK1::HIS3* pRS316-RSP5 [URA3 RSP5] pRS316-CTK1 [URA3 CTK1]	This study
YKS1420	*RSP5* shuffle *DST1* shuffle	W303	*RSP5::HIS3 DST1::HIS3* pRS316-RSP5 [URA3 RSP5] pRS316-DST1 [URA3 DST1]	This study
YKS1421	*RSP5* shuffle *THO2* shuffle	W303	*RSP5::HIS3 THO2::KANMX6* pRS316-RSP5 [URA3 RSP5] pRS316-THO2 [URA3 THO2]	This study
YKS1423	*RSP5* shuffle *BUR2* shuffle	W303	*RSP5::HIS3 BUR2::KANMX6* pRS316-RSP5 [URA3 RSP5] pRS316-BUR2 [URA3 BUR2]	This study
YKS1428	*Δdef1 CTK1* shuffle	W303	*DEF1:: KANMX6 CTK1::HIS3* pRS316-CTK1 [URA3 CTK1]	This study
YKS1430	*Δdef1 DST1* shuffle	W303	*DEF1:: KANMX6 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1432	*Δdef1 THO2* shuffle	W303	*DEF1:: KANMX6 THO2::KANMX6* pRS316-THO2 [URA3 THO2]	This study
YKS1434	*Δdef1 BUR2* shuffle	W303	*DEF1:: KANMX6 BUR2::KANMX6* pRS316-BUR2 [URA3 BUR2]	This study
YKS1749	*Δelc1 DST1* shuffle	W303	*ELC1:: KANMX6 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1605	*Δubp3 CTK1* shuffle	W303	*UBP3:: KANMX6 CTK1::HIS3* pRS316-CTK1 [URA3 CTK1]	This study
YKS1607	*Δubp3 DST1* shuffle	W303	*UBP3:: KANMX6 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1609	*Δubp3 THO2* shuffle	W303	*UBP3::HIS3 THO2::KANMX6* pRS316-THO2 [URA3 THO2]	This study
YKS1611	*Δubp3 BUR2* shuffle	W303	*UBP3::HIS3 BUR2::KANMX6* pRS316-BUR2 [URA3 BUR2]	This study
YKS1753	*Δubp2 Δdst1*	W303	*UBP2:: KANMX6 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1720	*Δubp4 DST1* shuffle	W303	*UBP4:: KANMX6 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1722	*Δubp6 DST1* shuffle	W303	*UBP6:: KANMX6 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1724	*Δubp10 DST1* shuffle	W303	*UBP10:: KANMX6 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1726	*Δubp12 DST1* shuffle	W303	*UBP12:: KANMX6 DST1::HIS3* pRS316-DST1 [URA3 DST1]	This study
YKS1826	SUB280 (wt)	BY		(Spence,

		4741		Sadis et al. 1995)
YKS1827	SUB413 (63R)	BY 4741		(Spence, Sadis et al. 1995)
YKS1856	*DST1* shuffle SUB280	BY 4741	*DST1::KANMX6* pRS316-DST1 [URA3 DST1]	This study
YKS1857	*DST1* shuffle SUB413	BY 4741	*DST1::KANMX6* pRS316-DST1 [URA3 DST1]	This study

4.2. PLASMIDS

Table 5 | Plasmids used in this study.

Nr.	*Name*	*Description*
Plasmids previously described		
pKS1	pBluescript II KS (+)	(Alting-Mees, Sorge et al. 1992)
pKS5	pRS313	(Sikorski and Hieter 1989)
pKS6	pRS314	(Sikorski and Hieter 1989)
pKS7	pRS315	(Sikorski and Hieter 1989)
pKS8	pRS316	(Sikorski and Hieter 1989)
pKS13	pASZ11	(Stotz and Linder 1990)
pKS14	Ydp-L	(Berben, Dumont et al. 1991)
pKS15	Ydp-H	(Berben, Dumont et al. 1991)
pKS84	pBS1479	(Puig, Rutz et al. 1998)
pKS85	pBS1539	(Puig, Rutz et al. 1998)
pKS82	pFA6a-*HIS3*MX6-P*GAL1*	(Longtine, McKenzie et al. 1998)
pKS595	pFA6a-*KAN*MX6-P*GAL1*	(Longtine, McKenzie et al. 1998)
pKS391	pRS315-*CTK1*	(Strasser and Hurt 2000)
pKS491	pRS316-CTK1	(Rother and Strasser 2007)
pKS621	pRS315-DST1	K. Sträßer (unpublished work)
pKS622	pRS316-DST1	K. Sträßer (unpublished work)
pKS181	pRS315-*THO2*	Gift from A. Aguilera
pKS520	pRS316-THO2	K. Sträßer (unpublished work)
pKS395	pRS315-BUR2	K. Sträßer (unpublished work)
pKS431	pRS316-BUR2	K. Sträßer (unpublished work)
pKS432	GHB232 [pRS315-Rpb1_CTD(14)WT Repeats]	(West and Corden 1995)
pKS433	GHB 233 [pRS315-Rpb1_CTD(7)WT(7)A5 Repeats]	(West and Corden 1995)
pKS434	GHB 234 [pRS315-Rpb1_CTD(9)WT(6)A2 Repeats]	(West and Corden 1995)
pKS435	pRP112	(Nonet, Sweetser et al. 1987)
pKS437	GHB83 [pRS315-Rpb1_CTD(13 3/7)WT Repeats]	(Nonet and Young 1989)
pKS438	GHB84 [pRS315-Rpb1_CTD(11 2/7)WT Repeats]	(Nonet and Young 1989)
pKS439	GHB200 [pRS315-Rpb1_CTD(10 5/7)WT Repeats]	(Nonet and Young 1989)
pKS759	pSE362-PRE1	Gift from S. Jentsch
pKS760	pSE362-pre1-1	Gift from S. Jentsch
pKS768	pBSIIKS+-5'-*Δctk1*-HIS3-3'	(Rother and Strasser 2007)

pKS1341	YEP112-Met25promoter-UBI4-6HIS	Gift from Stephan Jentsch
pKS 1342	YEP112-Adh1promoter-UBI4-6HIS	Gift from Stephan Jentsch
pKS1343	YEP181-Met25promoter-UBI4-6HIS	Gift from Stephan Jentsch
pKS1344	YEP181-Adh1promoter-UBI4-6HIS	Gift from Stephan Jentsch
pKS1345	Ubi-HIs	Gift from Stephan Jentsch
pKS1379	pRP214 (RPB2wt)	Plasmid was rescued from YKS1466 (Scafe, Martin et al. 1990)
pKS1380	pRP2-4L (*rpb2-4*)	Plasmid was rescued from YKS1467 (Scafe, Martin et al. 1990)
pKS1381	pRP2-7L (*rpb2-7*)	Plasmid was rescued from YKS1468 (Scafe, Martin et al. 1990)
pKS1382	pRP2-10L (*rpb2-10*)	Plasmid was rescued from YKS1469 (Scafe, Martin et al. 1990)
pKS1383	pRS414-*RSP5*	(Wang, Yang et al. 1999)
pKS1384	pRS414-*rsp5-1*	(Wang, Yang et al. 1999)
pKS1391	pRS415-RPB1K330R	(Chen, Ding et al. 2009)
pKS1392	pRS415-RPB1K695R	(Chen, Ding et al. 2009)
pKS1569	pJH80	(Hanna, Meides et al. 2007)
pKS1570	pJH81	(Hanna, Meides et al. 2007)
pKS1571	pUB146	(Hanna, Meides et al. 2007)
pRS Series		
pKS753	pRS314-*CIM3*	CIM3 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating BamHI and SalI sites and cloned into the same sites of pRS314;
pKS754	pRS315-*CIM3*	CIM3 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating BamHI and SalI sites and cloned into the same sites of pRS315;
pKS755	pRS316-*CIM3*	CIM3 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating BamHI and SalI sites and cloned into the same sites of pRS316;
pKS756	pRS314-*CIM5*	CIM5 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating BamHI and SalI sites and cloned into the same sites of pRS314;
pKS757	pRS315-*CIM5*	CIM5 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating BamHI and SalI sites and cloned into the same sites of pRS315;
pKS758	pRS316-*CIM5*	CIM5 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating BamHI and SalI sites and cloned into the same sites of pRS316;
pKS1309	pRS314-*UBC4*	UBC4 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and XhoI sites and cloned into the same sites of pRS314;
pKS1310	pRS315-*UBC4*	UBC4 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and

		XhoI sites and cloned into the same sites of pRS315;
pKS1311	pRS316-*UBC4*	UBC4 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and XhoI sites and cloned into the same sites of pRS316;
pKS1312	pRS314-*UBC5*	UBC5 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and XhoI sites and cloned into the same sites of pRS314;
pKS1313	pRS315-*UBC5*	UBC5 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and XhoI sites and cloned into the same sites of pRS315;
pKS1314	pRS316-*UBC5*	UBC5 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and XhoI sites and cloned into the same sites of pRS316;
pKS1315	pRS314-*RSP5*	RSP5 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and XhoI sites and cloned into the same sites of pRS314;
pKS1316	pRS315-*RSP5*	RSP5 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and XhoI sites and cloned into the same sites of pRS315;
pKS1317	pRS316-*RSP5*	RSP5 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and XhoI sites and cloned into the same sites of pRS316;
pKS1334	pRS315-*PRE1*	PRE1 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and SalI sites and cloned into the same sites of pRS315;
pKS1335	pRS316- *PRE1*	PRE1 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and SalI sites and cloned into the same sites of pRS316;
pKS1336	pRS314-*PRE2*	PRE2 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and XhoI sites and cloned into the same sites of pRS314;
pKS1337	pRS315-*PRE2*	PRE2 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and XhoI sites and cloned into the same sites of pRS315;
pKS1338	pRS316-*PRE2*	PRE2 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and XhoI sites and cloned into the same sites of pRS316;
pKS1377	pRS316-*RPA12*	RPA12 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and SalI sites and cloned into the same sites of pRS316;
pKS1385	pRS315-*SUG2*	SUG2 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and SalI sites and cloned into the same sites of pRS315;
pKS1389	pRS314-*PRE1*	The EcoRI-SalI fragment of pKS759 was subcloned into the same sites of pRS314 (pKS6)
pKS1390	pRS314-*pre1-1*	The EcoRI-SalI fragment of pKS760 was subcloned into the same sites of pRS314 (pKS6)
pKS1397	pRS315-RPB1	The NotI-SalI fragment of pBSIIKS+-RPB1 (pKS1393) was subcloned into the corresponding sites of pRS315 (pKS7)
pKS1398	pRS315-RPB1K330R	The NotI-SalI fragment of pBSIIKS+-RPB1K330R (pKS1394) was subcloned into the corresponding sites of pRS315 (pKS7)
pKS1399	pRS315-RPB1K695R	The NotI-SalI fragment of pBSIIKS+-RPB1K695R

		(pKS1395) was subcloned into the corresponding sites of pRS315 (pKS7)
pKS1400	pRS315-RPB1K330R_K695R	The NotI-SalI fragment of pBSIIKS+-RPB1K330R_K695R (pKS1396) was subcloned into the corresponding sites of pRS315 (pKS7)
Disruption cassettes		
pKS1323	pBSIIKS+-5'-Δubc4-3'	about 500 bp 5' and 3' of the UBC4 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1324	pBSIIKS+-5'-Δubc4-HIS3-3'	about 500 bp 5' and 3' of the UBC4 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the HIS3 marker was inserted; used for disruption of UBC4 with HIS3 marker;
pKS1325	pBSIIKS+-5'-Δubc5-3'	about 500 bp 5' and 3' of the UBC5 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1326	pBSIIKS+-5'-Δubc5-HIS3-3'	about 500 bp 5' and 3' of the UBC5 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the HIS3 marker was inserted; used for disruption of UBC5 with HIS3 marker
pKS1327	pBSIIKS+-5'-Δrsp5-3'	about 500 bp 5' and 3' of the RSP5 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1328	pBSIIKS+-5'-Δrsp5-HIS3-3'	about 500 bp 5' and 3' of the RSP5 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the HIS3 marker was inserted; used for disruption of RSP5 with HIS3 marker
pKS1386	pBSIIKS+-5'-Δctk1-KANMX6-3'	the KANMX6 marker was inserted into this BamHI site of pKS766; used for disruption of CTK1 with KANMX6 marker
pKS1349	pBSIIKS+-5'-Δdst1-3'	about 500 bp 5' and 3' of the DST1 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1350	pBSIIKS+-5'-Δdst1-HIS3-3'	about 500 bp 5' and 3' of the DST1 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the HIS3 marker was inserted; used for disruption of DST1 with HIS3 marker
pKS1387	pBSIIKS+-5'-Δdst1-LEU2-3'	the LEU2 marker was inserted into this BamHI site of pKS1349; used for disruption of DST1 with LEU2 marker
pKS1388	pBSIIKS+-5'-Δdst1-KANMX6-3'	the KANMX6 marker was inserted into this BamHI site of pKS1349; used for disruption of DST1 with KANMX6 marker
pKS1351	pBSIIKS+-5'-Δtho2-3'	about 500 bp 5' and 3' of the THO2 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1352	pBSIIKS+-5'-Δtho2-KANMX6-3'	about 500 bp 5' and 3' of the THO2 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this

		BamHI site the KANMX6 marker was inserted; used for disruption of THO2 with KANMX6 marker
pKS1353	pBSIIKS+-5'-Δtho2-KANMX6-3'	about 500 bp 5' and 3' of the THO2 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the KANMX6 marker was inserted; used for disruption of THO2 with KANMX6 marker
pKS1401	pBSIIKS+-5'-Δtho2-LEU2-3'	the LEU2 marker was inserted into this BamHI site of pKS1351; used for disruption of THO2 with LEU2 marker
pKS1402	pBSIIKS+-5'-Δtho2-HIS3-3'	the HIS3 marker was inserted into this BamHI site of pKS1351; used for disruption of THO2 with HIS3 marker
pKS1348	pBSIIKS+-5'-Δbur2-KANMX4-3'	Disruption cassette of the BUR2 ORF was amplified by PCR from genomic DNA of BUR2::KANMX4 disrupted BY strain bought from Euroscarf
pKS1403	pBSIIKS+-5'-Δbur2-HIS3-3'	the HIS3 marker was exchanged with the KANMX6 cassette of pKS1348 using the BamHI site; used for disruption of BUR2 with HIS3 marker
pKS1404	pBSIIKS+-5'-Δbur2-LEU2-3'	the LEU2 marker was exchanged with the KANMX6 cassette of pKS1348 using the BamHI site; used for disruption of BUR2 with LEU2 marker
pKS1357	pBSIIKS+-5'-Δpdr5-3'	about 500 bp 5' and 3' of the PDR5 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1346	pBSIIKS+-5'-Δpdr5-HIS3-3'	about 500 bp 5' and 3' of the PDR5 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the HIS3 marker was inserted; used for disruption of PDR5 with HIS3 marker
pKS1368	pBSIIKS+-5'-Δrpa12-3'	about 500 bp 5' and 3' of the RPA12 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1369	pBSIIKS+-5'-Δrpa12-HIS3-3'	about 500 bp 5' and 3' of the RPA12 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the HIS3 marker was inserted; used for disruption of RPA12 with HIS3 marker
pKS1370	pBSIIKS+-5'-Δrpa12-KANMX6-3'	about 500 bp 5' and 3' of the RPA12 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the KANMX6 marker was inserted; used for disruption of RPA12 with KANMX6 marker
pKS1374	pBSIIKS+-5'-Δubp3-3'	about 500 bp 5' and 3' of the UBP3 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1375	pBSIIKS+-5'-Δubp3-HIS3-3'	about 500 bp 5' and 3' of the UBP3 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the HIS3 marker was inserted; used for disruption of UBP3 with HIS3 marker
pKS1376	pBSIIKS+-5'-Δubp3-KANMX6-3'	about 500 bp 5' and 3' of the UBP3 ORF were amplified by PCR and cloned into pBluescriptIIKS+

		creating a BamHI site "between them"; into this BamHI site the KANMX6 marker was inserted; used for disruption of UBP3 with KANMX6 marker
pKS1553	pBSIIKS+-5'-Δubp2-3'	about 500 bp 5' and 3' of the UBP2 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1554	pBSIIKS+-5'-Δubp2-KANMX6-3'	about 500 bp 5' and 3' of the UBP2 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the KANMX6 marker was inserted; used for disruption of UBP2 with KANMX6 marker
pKS1555	pBSIIKS+-5'-Δubp4-3'	about 500 bp 5' and 3' of the UBP4 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1556	pBSIIKS+-5'-Δubp4-KANMX6-3'	about 500 bp 5' and 3' of the UBP4 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the KANMX6 marker was inserted; used for disruption of UBP4 with KANMX6 marker
pKS1557	pBSIIKS+-5'-Δubp6-3'	about 500 bp 5' and 3' of the UBP6 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1559	pBSIIKS+-5'-Δubp6-KANMX6-3'	about 500 bp 5' and 3' of the UBP6 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the KANMX6 marker was inserted; used for disruption of UBP6 with KANMX6 marker
pKS1560	pBSIIKS+-5'-Δubp10-3'	about 500 bp 5' and 3' of the UBP10 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1562	pBSIIKS+-5'-Δubp10-KANMX6-3'	about 500 bp 5' and 3' of the UBP10 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the KANMX6 marker was inserted; used for disruption of UBP10 with KANMX6 marker
pKS1563	pBSIIKS+-5'-Δubp12-3'	about 500 bp 5' and 3' of the UBP12 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1565	pBSIIKS+-5'-Δubp12-KANMX6-3'	about 500 bp 5' and 3' of the UBP12 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the KANMX6 marker was inserted; used for disruption of UBP12 with KANMX6 marker
pKS1551	pBSIIKS+-5'-Δelc1-3'	about 500 bp 5' and 3' of the ELC1 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1552	pBSIIKS+-5'- Δelc1-KANMX6-3'	about 500 bp 5' and 3' of the ELC1 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the KANMX6 marker was inserted; used for disruption of ELC1 with KANMX6 marker
pKS1576	pBSIIKS+-5'-Δrad26-3'	about 500 bp 5' and 3' of the RAD26 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"

pKS1577	pBSIIKS+-5'- Δ rad26-KANMX6-3'	about 500 bp 5' and 3' of the RAD26 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the KANMX6 marker was inserted; used for disruption of RAD26 with KANMX6 marker
pKS1578	pBSIIKS+-5'-Δasr1-3'	about 500 bp 5' and 3' of the ASR1 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1579	pBSIIKS+-5'- Δasr1-KANMX6-3'	about 500 bp 5' and 3' of the ASR1 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the KANMX6 marker was inserted; used for disruption of ASR1 with KANMX6 marker
pKS1580	pBSIIKS+-5'-Δbre1-3'	about 500 bp 5' and 3' of the BRE1 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"
pKS1581	pBSIIKS+-5'- Δbre1-KANMX6-3'	about 500 bp 5' and 3' of the BRE1 ORF were amplified by PCR and cloned into pBluescriptIIKS+ creating a BamHI site "between them"; into this BamHI site the KANMX6 marker was inserted; used for disruption of BRE1 with KANMX6 marker
pBluescript Series		
pKS1329	pBS1479minusALL	pBS1479 (pKS84) was digested with SmaI and KpnI; the ~3.4kb fragment was blunt ended and then ligated
pKS1330	pBS1479HIS3	pBS1479minusALL (pKS1329) was digested with PstI; the linearised plasmid was blunt ended and then dephosphorylated with CIAP; the HIS3 cassette was isolated from YDp-H after a BamHI digestion and was also blunt ended; the two fragments were then ligated to create pBS1479HIS3
pKS1331	pBS1479KANMX6	pBS1479minusALL (pKS1329) was digested with PstI; the linearised plasmid was blunt ended and then dephosphorylated with CIAP; the KANMX6 cassette was isolated from pKS935 after a BamHI digestion and was also blunt ended; the two fragments were then ligated to create pBS1479KANMX6
pKS1332	pBS1479LEU2	pBS1479minusALL (pKS1329) was digested with PstI; the linearised plasmid was blunt ended and then dephosphorylated with CIAP; the LEU2 cassette was isolated from YDp-L after a BamHI digestion and was also blunt ended; the two fragments were then ligated to create pBS1479LEU2
pKS1333	pBS1479LEU2	pBS1479minusALL (pKS1329) was digested with PstI; the linearised plasmid was blunt ended and then dephosphorylated with CIAP; the LEU2 cassette was isolated from YDp-L after a BamHI digestion and was also blunt ended; the two fragments were then ligated to create pBS1479LEU2
pKS1393	pBSIIKS+-RPB1	RPB1 + ca. 500 bp promoter and ca. 300 bp terminator was amplified by PCR creating NotI and SalI sites and cloned into the same sites of pBSIIKS(+); Midi was extensively sequenced

pKS1394	pBSIIKS+-RPB1K330R	a Lysine →Arginine Mutation at aa 330 in Rpb1 was introduced by quick change mutagenesis into pBSIIKS+-RPB1 (pKS1393); Midi was extensively sequenced
pKS1395	pBSIIKS+-RPB1K695R	a Lysine →Arginine Mutation at aa 695 in Rpb1 was introduced by quick change mutagenesis into pBSIIKS+-RPB1 (pKS1393); Midi was extensively sequenced
pKS1396	pBSIIKS+-RPB1K330R_K695R	An additional Lysine →Arginine Mutation at aa 695 in Rpb1 was introduced by quick change mutagenesis into pBSIIKS+-RPB1K330R (pKS1394); Midi was extensively sequenced
Miscellaneous		
pKS1339	pASZ11-*PRE1*	The EcoRI-SalI fragment of pKS759 was subcloned into the same sites of pASZ11 (pKS13)
pKS1340	pASZ11-*pre1-1*	The EcoRI-SalI fragment of pKS760 was subcloned into the same sites of pASZ11 (pKS13)
pKS1347	pYM46-NatNT3	The NcoI-EcoRV fragment of pYM21 (pKS689) was inserted into the corresponding sites of pYM46 (pKS712)

4.3. OLIGONUCLEOTIDES

Table 6 | Oligonucleotides used in this study.

Purpose	*Name*	*Sequence (5'-3')*
Gene disruptions		
Disruption of *DST1*	5'_NotI_Dst1_prom	gggGCGGCCGCGCATTCACACCGCTCTGCCTATTCA
	3'_BamHI_Dst1_prom	gggGGATCCGATGAATGCTCTTATGCGGACTGAC
	5'_BamHI_Dst1_term	gggGGATCCGATGAGAGTAGCCACGGACAGAGC
	3'_XhoI_Dst1_ term	gggCTCGAGACTCCTGGTGTGATTAGTGGTAGA
Disruption of *THO2*	5'_NotI_Tho2_prom	gggGCGGCCGCCAGTCCGATGTACTTTTGTT
	3'_BamHI_Tho2_prom	gggGGATCCAAAGTCCTGAAAATGTATAC
	5'_BamHI_Tho2_term	gggGGATCCTTACTAGTACTTAACATACC
	3'_XhoI_Tho2_ term	gggCTCGAGTATTCGCATTAGTACCATTT
Disruption of *BUR2*	5'_EcoRV_Bur2_prom	ggg GATATCCTCGGATAAAACTTCCTTAAC
	3'_XhoI_Bur2_ term	gggCTCGAGCCGAAGATAATGACTGTTC
Disruption of *PDR5*	5'_NotI_Pdr5_prom	gggGCGGCCGCTTCCGCGGAATCGCTCATGCCGCG G
	3'_BamHI_Pdr5_prom	gggGGATCCGTCTAAAGTCTTTCGAACGAGC
	5'_BamHI_Pdr5_term	gggGGATCCTGGTTAAGAAAAGAAACTTACCA
	3'_XhoI_Pdr5_ term	gggCTCGAGCACGTTCGTTGTACTTCCAGTCG
Disruption of *UBC4*	5'_NotI_Ubc4_prom	gggGCGGCCGCAGGGTAACTGCACTATTCAT
	3'_BamHI_Ubc4_prom	gggGGATCCGTTTTTTTGGATGCTTGTTT
	5'_BamHI_Ubc4_term	gggGGATCCACAGAAGTCCTTACTCAGCT
	3'_XhoI_Ubc4_ term	gggCTCGAGCTCCATACTGTTCGAGGAAA
Disruption of *UBC5*	5'_NotI_Ubc5_prom	gggGCGGCCGCTATATGAAATCATCTGCAAC
	3'_BamHI_Ubc5_prom	gggGGATCCTTTTAGTTTAGTTGAGGGTG
	5'_BamHI_Ubc5_term	gggGGATCCTTAATTTGGGCTAACGGATA
	3'_XhoI_Ubc5_ term	gggCTCGAGCCCATCGTGAGTGCTTTGTT
Disruption of *RSP5*	5'_NotI_Rsp5_prom	gggGCGGCCGCATCGCATCTGCTAATTGATA
	3'_BamHI_Rsp5_prom	gggGGATCCCTTTTTTCTTTCCTTTCTGTTAC

	5'_BamHI_Rsp5_term	gggGGATCCTTATTCCGCACCAATTTTTT
	3'_XhoI_Rsp5_ term	gggCTCGAGGGTATCATGCTCCTTTTGAA
Disruption	5'_NotI_Ubp3_prom	gggGCGGCCGCTATGCCATTGAAGGGCGGCT
of *UBP3*	3'_BamHI_Ubp3_prom	gggGGATCCTTTTTTTAATGATGATGGAA
	5'_BamHI_Ubp3_term	gggGGATCCGACGGGGGGTGGTATTATAG
	3'_XhoI_Ubp3_ term	gggCTCGAGATTCCATTGTTGCCAAAGGC
Disruption	5'_NotI_Ubp2_prom	gggGCGGCCGCATCTAGACACCGCTATCAAG
of *UBP2*	3'_BamHI_Ubp2_prom	gggGGATCCTTCCTTATACCTTCTTAACC
	5'_BamHI_Ubp2_term	gggGGATCCTCTTAGTCAATGAAGAG
	3'_XhoI_Ubp2_ term	gggGTCGACCGACGTTATTGCTGATTTGC
Disruption	5'_NotI_Ubp4_prom	gggGCGGCCGCGGTTGGACACTTTGGAACAC
of *UBP4*	3'_BamHI_Ubp4_prom	gggGGATCCAACTTAAGCATGTATCAAATAG
	5'_BamHI_Ubp4_term	gggGGATCCTTCATTTGAATAAATAACTG
	3'_XhoI_Ubp4_ term	gggCTCGAGGATGATGACTGGGAATGAGTG
Disruption	5'_NotI_Ubp6_prom	gggGCGGCCGCCGGTGGTGTCTTAATGGTTTC
of *UBP6*	3'_BamHI_Ubp6_prom	gggGGATCCATTTTAACACAAGGATAGGTAG
	5'_BamHI_Ubp6_term	gggGGATCCGTCCATTTTTCATTTTTTC
	3'_XhoI_Ubp6_ term	gggCTCGAGGGCAGCATTTCTATAAAGGC
Disruption	5'_NotI_Ubp10_prom	gggGCGGCCGCGTAGTGAAGAATTGTCCTG
of *UBP10*	3'_BamHI_Ubp10_pro	gggGGATCCAGTCTGTGATTGTGATATGAC
	5'_BamHI_Ubp10_term	gggGGATCCAAAAACTCGATATTCCTGG
	3'_XhoI_Ubp10_ term	gggCTCGAGGTACCGTCAATTTCGAGAA
Disruption	5'_NotI_Ubp12_prom	gggGCGGCCGCCAATGTCACTGCCATCTCTAAAG
of *UBP12*	3'_BamHI_Ubp12_pro	gggGGATCCTTTCGAATGAAGAAACCTTCG
	5'_BamHI_Ubp12_term	gggGGATCCTGACATGAGTTTATATGATAG
	3'_SalI_Ubp12_ term	gggGTCGACCTAGTGGTAAGAAGAAGATC
Disruption	5'_NotI_Elc1_prom	gggGCGGCCGCAGTTGGCCATTTTAATTCGC
of *ELC1*	3'_BamHI_ Elc1_prom	gggGGATCCTTAGTTATGGTTTTTTATTC
	5'_BamHI_ Elc1_term	gggGGATCCGTACAGGAAAAACAAAAAAG
	3'_SalI_ Elc1_ term	gggGTCGACATTCTTACATTTTATGCGGC
Disruption	5'_NotI_Rad26_prom	gggGCGGCCGCGAATCGTCGACATGACTAAG
of *RAD26*	3'_BamHI_ Rad26_pro	gggGGATCCCACTGATGTATGTTTCTCTG
	5'_BamHI_ Rad26_term	gggGGATCCAAACTTAGAATGGACAGAGTAC
	3'_XhoI_ Rad26_ term	gggCTCGAGCAACGGGTATCTCTACTATC
Disruption	5'_NotI_Rpa12_prom	gggGCGGCCGCGCACTTTTCGTAACTTTATC
of *RPA12*	3'_BamHI_Rpa12_prom	gggGGATCCTCTTATAAGTACTAACCTGG
	5'_BamHI_Rpa12_term	gggGGATCCTTGGGTTGTGTTCGCACATATTAC
	3'_SalI_Rpa12_term	gggGTCGACGTTTGTAGCTCTTTATCACGC
Disruption	5'_NotI_Asr1_prom	gggGCGGCCGCACAGCCATCTTTATCTTTAC
of *ASR1*	3'_ BamHI_Asr1_prom	gggGGATCCCTTTTTATATGATATTTGG
	5'_BamHI _Asr1_term	gggGGATCCTAATGCAATATAACATTAAAACAAG
	3'_ XhoI _Asr1_term	gggCTCGAGGGAACTCATATATTATCTCTTC
Disruption	5'_NotI_Bre1_prom	gggGCGGCCGCTAGTTGATTATGTATGGCAG
of *BRE1*	3'_ BamHI_Bre1_prom	gggGGATCCATCTGATTATTATCAGCTAG
	5'_BamHI_Bre1_term	gggGGATCCTACTAATTATTCTACCAC
	3'_ SalI_Bre1_term	gggGTCGACATATATCTTGAACAAGC
Checking	5'_HIS_del_down	CTATACGTGTCATTCTGAACGAGGCGC
for correct	3'_HIS_del_up	GGGAAAGGACTGTGTTATGACTTCC
integra-	5'_Kan_del_down	GCAGTTTCATTTGATGCTCGATGAG
tion after	3'_Kan_del_up	GAAACGTGAGTCTTTTCCTTACCC
gene	5'_Ctk1_600_up_del	CCAAAGATTTACGACAACTA
disruption	5'_DstI_600_up_del	CGAACATCATTTTCAAATTGATCA
	3'_DstI_400_down_del	TCTGCACCTTTATGTGTGCATCTA
	5'_Pdr5_600_up_del	ACGATTATCACGACACAACCTTGC

	3'_Pdr5_400_down_del	GTATGTCAGCCTGCCGTATGTCA
	5'_Ubc4_600_up_del	GCTACTGTTTTAGTCAACAT
	3'_Ubc4_400_down_del	AGGTACGGTTAACGTTGACG
	5'_Ubc5_600_up_del	TCTTAGATTATGTTGGGCAA
	3'_Ubc5_400_down_del	ATTTAGCTAGATGAATCAGA
	5'_Rsp5_600_up_del	AAAGACCAAATTGCAGTGAT
	3'_Rsp5_400_down_del	TATGGTTGTAAAAAGCAGGG
	5'_Tho2_600_up_del	ATGAAACGTACTCCAAAGAT
	3'_Tho2_400_down_del	ATGAAACATGGGGTCGTATC
	5'_Ubp3_600_up_del	ATGCATTGCACATCTGAGAT
	3'_Ubp3_400_down_del	GCTAAATTTGGATGATTAC
	5'_Ubp2_600_up_del	CAATTTAGAAAGAACAGATGG
	3'_Ubp2_400_down_del	GTATCATCATTTCCATTGAC
	5'_Ubp4_600_up_del	CTTTCTCAATCGTCACTAGG
	3'_Ubp4_400_down_del	CGAAAGTTGCTGTGTCTCAAG
	5'_Ubp6_600_up_del	GCATTGTGTTTAAATAATCCAC
	3'_Ubp6_400_down_del	CAAATCGAGTCGTTGAAC
	5'_Ubp10_600_up_del	CAATAGTGTTGAAGACCCAC
	3'_Ubp10_400_down_d	GGCATAGTTTATTGGAGATG
	5'_Ubp12_600_up_del	AGAAGTTGGTGACAAGCTTG
	3'_Ubp12_400_down_d	GCAGCCATTCTTACATCCTAC
	5'_Elc1_600_up_del	TTCACAATTTTGATGGAGC
	3'_Elc1_400_down_del	AATTGAAAAGGCACAGACAG
	5'_Rad26_600_up_del	ACCCTCAAGAATGTCTTGAC
	3'_Rad26_400_down_d	GAATCATCGGAGAAGCCA
	5'_Rpa12_600_up_del	CTTTCTTAGTAACATAAGG
	3'_Rpa12_400_down_d	ACAATGGCGTTCGACAGAT
	5'_ Asr1_600_up_del	AGTACCATGGGTTATAAATG
	3'_ Asr1_400_down_del	AAAGGGTTGTCGACATGTAG
	5'_ Bre1_600_up_del	TTGATAAACCTGTTCAAC
	3'_ Bre1_400_down_del	CAGTAGAAGAGTTATGTTAC
TAP-tagging		
Carboxyl-Terminal TAP tagging of proteins	Pre1 primer 1	AAATCGTGGATAAAGATGGCATAAGACAAGTAGATG ACTTCCAGGCACAGTCCATGGAAAAGAGAAG
	Pre1 primer 2	ATTTAATTTTATGAACGAGGAAGATAATTACTTTAG TATATCATTAGCAATACGACTCACTATAGGG
	Pre2 primer 1	TATTTTGGAAGGTCAAGGAAGAGGAAGGATCTTTCA ACAACGTTATTGGCTCCATGGAAAAGAGAAG
	Pre2 primer 2	GGACTTCCTTCATAATTCTATGGGAAGCCATATTGG ATCCTTGAAGAGAATACGACTCACTATAGGG
	Pre4 primer 1	TGAAATGGGACTTCGCCAAGGATATTAAAGGCTACG GTACTCAAAAAATTTCCATGGAAAAGAGAAG
	Pre4 primer 2	GAAGAGAGAAGAGTGATGATATTATGAATTGAAAAA TAAAAATAAAATGATACGACTCACTATAGGG
	Pup1 primer 1	ATTGTCAATATTTGTGACATACAAGAAGAACAAGTC GATATAACGGCTTCCATGGAAAAGAGAAG
	Pup1 primer2	TATGTAAATAGTTTTGCGGTTAGTTTTTGACTTCCT TTATCACATTTTGTTACGACTCACTATAGGG
	Sug1 primer 1	TTATGAACAAGAACCAAGAAACGGCCATTTCTGTCG CCAAGCTGTTCAAGTCCATGGAAAAGAGAAG
	Sug1 primer 2	GTATATCCAGCGGTATAATTTTGCCTCTTAGTTAAT GCTAAACTATGATATACGACTCACTATAGGG
	Sug2 primer 1	AAGTAGCTGAAGTTAAGAAATTGGAAGGCACTATAG AATACCAAAAATTATCCATGGAAAAGAGAAG

	Sug2 primer 2	CTAGAGTTCAATAGCCATTTCGAGCTTAAATAAGGC AAGCTTTCTCGCATTACGACTCACTATAGGG
	Cim5 primer 1	TTAGCGGATACAAGAAGTTTAGTTCCACATCGCGTT ATATGCAATATAATTCCATGGAAAAGAGAAG
	Cim5 primer 2	GAGGGGATACTCTCGTATCCTTTTTATCTCGATAGT TGTAGTTGTACTTGTACGACTCACTATAGGG
	Rpb1 primer 1	CTCCAAAGCAAGACGAACAAAAGCATAATGAAAATGA AAATTCCAGATCCATGGAAAAGAGAAG
	Rpb1 primer 2	CCCTATCCCTACCATAATGCTATGAAAAATAATGGT ATATTTGGTATACGTACGACTCACTATAGGG
	Rpb2 primer 1	GGCTATGAACATTACACCACGTTTATATACCGATCG TTCGAGAGATTTTTCCATGGAAAAGAGAAG
	Rpb2 primer 2	GAAATGCTTCATTCTTATTTTATTACTATTGCCTAC TTGTTTACCCTACGACTCACTATAGGG
	Rpb3 primer 1	ATGCATCTCAAATGGGTAATACTGGATCAGGAGGG TATGATAATGCTTGGTCCATGGAAAAGAGAAG
	Rpb3 primer 2	TAAAGCTTTTTTTCTCTTATTATTTTCGGTTCGTTC ACTTGTTTTTTTTCTACGACTCACTATAGGG
	Rpa190 primer 1	ATGTTGGTACGGGTTCATTTGATGTGTTAGCAAAG GTTCCAAATGCGGCTTCCATGGAAAAGAGAAG
	Rpa190 primer 2	AATGGCGCTACCCATTGTGCATTTTTCTCTCTTTTC TTCTGACCTTCTCCTACGACTCACTATAGGG
	Rpc160 primer 1	GCGATGTCTATTTGAAAGTCTCTCAAATGAGGCAGC TTTAAAAGCGAACTCCATGGAAAAGAGAAG
	Rpc160 primer 2	GGTTTTTATCATGTAGTTTTATATGTATAAATACGT TAAATGACTGTGGTAGTACGACTCACTATAGGG
Gene Cloning		
Cloning of genes into the pRSseries	5'_BamHI_Cim3_500	gggGGATCCCCACAGCCAAACTGGTCAATTGG
	3'_SalI_Cim3_300	gggGTCGACAGGCTGAGTGGTCGTTTGCTGTG
	5'_NotI_Sug2_500	gggGCGGCCGCAGGTCTATTACCGATGGGCAAAA
	3'_SalI_Sug2_300	gggGTCGACGGATTCTTCATTAGTTGGGACGA
	5'_BamHI_Cim5_500	gggGGATCCCGCCATTGTTGCACTATAAGGCG
	3'_SalI_Cim5_300	gggGTCGACCAATTAGAGAATGAGATTGAGGG
Gene Sequencing		
Rpb1	Rpb1_seq1	CTGAAATCATATCTGTGTTG
	Rpb1_seq2	CACCTGAAGAAGTTAGAGCA
	Rpb1_seq3	TCTGAAGATGATCCTACTCA
	K330R_Rpb1_seq 1	GGAAACACTAGAGCATAACG
	Rpb1_seq5	GCCCACAGAGTAAAAGTTAT
	K695R_Rpb1_seq 1	GTTTGTGCTAAGTTGTTTGG
	Rpb1_seq7	CGTGAAGGTCTTATCGATAC
	Rpb1_seq8	ATTAGTGTTGCGTGGTAAGA
	Rpb1_seq9	TCTCATTATTGGATGAAGAG
	Rpb1_seq10	ATGTTATTGCTTCTGATGGT
	Rpb1_seq11	ACATCTCCCGGATTTGGAGT
	Rpb1_seq12	TTCTCCAAAGCAAGACGAAC
Ubi4	Ubi4_seq1	TTAATGCGCCGCTACAGGGC
	Ubi4_seq2	ACTACCAACGCAATATGGAT
	Ubi4_seq3	ATCGATAACGTTAAGTCGAA
	Ubi4_seq4	GAACCTTGTCTGACTACAAC
Rsp5	Rsp5_seq1	GATACTGCTACATCGAGTGG
	Rsp5_seq2	CACGTGTATATTTCGTTGAC
	Rsp5_seq3	GTATAGATGGTGTCTTGG
Ubp6	Ubp6_seq1	ATACGAGATTTCGTTCTC

	Ubp6_seq2	AGCGATTCTAAATTAC
	Ubp6_seq3	TTGAATCTTTAGCCGGTG
Generation of GAL-Depletion Strains		
GAL1 promoter insertion	Dst1 F4	TCACTCGATGATGGGACTACGTATTGAAAAATATTG AATGAAAAATTACTGAATTCGAGCTCGTTTAAAC
	Dst1 R2	TTGTTCTTTTCTAGATTCTTAACATGTACCAGTACT TCCTTACTATCCATTTTGAGATCCGGGTTTT
	Tho2 F4	AATTATAGGGCTGTGATTTTATCGTCTTTATCTAAA GCATAATAGGGTGAGAATTCGAGCTCGTTTAAAC
	Tho2 R2	ACTTTTTGAGAAAGAGCGTTCAATTTGGAAAGTAGC GTCTGTTCTGCCATTTTGAGATCCGGGTTTT
	Bur2 F4	TATTTCTGTTAGAAAGCAAGTAGCTATTTTGATTGG TAATTATATACACAGAATTCGAGCTCGTTTAAAC
	Bur2 R2	GGTACAGCTTGAAATTTTTTCACGTCACCACTTGAA GATGTAGCAGACATTTTGAGATCCGGGTTTT
Checking for correct insertion of pGAL1	5'_colPCR_Gal1P	TGTAAAGAGCCCCATTATCT
	3'_Gal1P_Bur2_colPC	TGCACTCAAAAACCCACTGG
	3'Gal1P_Ctk1_colPCR	TGAGAAGATATATTGTCCAT
	3'Gal1P_Dst1_colPCR	GTTTGCTGATCTCTACATTA
	3'Gal1P_Tho2_colPCR	TTGACTCCAGTGCAGTGAAA
Real Time PCR primers		
RNApolII mRNA levels	5'_Rpb1_RT1	GTGTGGATTTTTCGGCAAGAA
	3'_Rpb1_RT1	TGGAACACCGACTTGGTCTAATT
	5'_Rpb3_RT1	CGAAGGTGACCCCTTCGAT
	3'_Rpb3_RT1	CGGGAATAGACCCCACAGATT
	5'_Adh1_M_RT2	AGCCGCTCACATTCCTCAAG
	3'_Adh1_M_RT2	ACGGTGATACCAGCACACAAGA
	5'_Actin_RT3	TCAGAGCCCCAGAAGCTTTG
	3'_Actin_RT3	TTGGTCAATACCGGCAGATTC
ChIP qPCR primers	5'_Adh1_prom_RT1	ACGACAAAGACAGCACCAACA
	3'_Adh1_prom_RT1	ACCCCTCATCAGCTCTGGAA
	5'_Adh1_5'gene_RT1	GTTGTCGGCATGGGTGAAA
	3'_Adh1_5'gene_RT1	GGCGTAGTCACCGATCTTCC
	5'_Adh1_M_RT2	AGCCGCTCACATTCCTCAAG
	3'_Adh1_M_RT2	ACGGTGATACCAGCACACAAGA
	5'_Adh1_3'gene_RT1	TTGGACTTCTTCGCCAGAGG
	3'_Adh1_3'gene_RT1	GCCGACAACCTTGATTGGAG
	5'_Pma1_M_RT2	AAATCTTGGGTGTTATGCCATGT
	3'_Pma1_M_RT2	CCAAGTGTCTAGCTTCGCTAACAG
	5'_Actin_RT3	TCAGAGCCCCAGAAGCTTTG
	3'_Actin_RT3	TTGGTCAATACCGGCAGATTC
	5'_YERTIR_RT	TGCGTACAAAAAGTGTCAAGAGATT
	3'_YERTIR_RT	ATGCGCAAGAAGGTGCCTAT

4.4. ANTIBODIES

Table 7 | Antibodies used in this study.

Name	*Source*	*Dilution*	*Company*
Primary Antibodies			
anti-Ser2P (H5)	mouse	1:500	MMS-129R; Covance
anti-Ser5P (H14)	mouse	1:500	MPY-127R; Covance

anti-RPB1 (8WG16)	mouse	1:500	MMS-126R; Covance
anti-RPB1 (yN-18)	goat	1:1000	sc-8952; Santa Cruz
anti-PGK1	mouse	1:20000	A-6457, Molecular Probes
Peroxidase Anti-Peroxidase (PAP)	rabbit	1:5000	P1292, Sigma
anti-DST1	goat	1:200	sc-26335; Santa Cruz
anti-MED2	goat	1:1000	sc-28058; Santa Cruz
anti-SPT5	goat	1:400	sc-26355; Santa Cruz
anti-mono/polyubiquitin (FK2)	mouse	1:1000	PW8810; Biomol
anti-polyubiquitin (FK1)	mouse	1:500	PW8805; Biomol
anti-ubiquitin (P4D1)	mouse	1:1000	#3936;Cell Signalling Tech.
anti-polyubiquitin (Apu2.07; K48-specific chains)	human	1:2000	(Newton, Matsumoto et al. 2008)
anti-polyubiquitin (Apu3.A8; K63-specific chains)	human	1:2000	(Newton, Matsumoto et al. 2008)
Secondary Antibodies			
anti-mouse-HPRO (IgG H+L)	goat	1:3000	#170-6516; Biorad
anti-goat-HPRO (IgG)	rabbit	1:5000	A5420; Sigma
anti-mouse-HPRO (IgM)	goat	1:300	A8786; Sigma
anti-human-HPRO (IgG)	goat	1:2000	#55220; Cappel

4.5. CHEMICALS & CONSUMABLES

Table 8 | Chemicals and consumables used in this study.

Name	*Source*
Standard chemicals and consumables	
Standard chemicals and consumables were purchased from the companies listed	Acros Organics; Applichem; Applied Biosciences; Apollo Scientific Limited; Axon; Becton Dickinson; Beckman Coulter; Biaffin; Biomol; Biorad; Biozym; Fermentas; Formedium; GE Healthcare; Gilson; Invitrogen; Lake Placid Biologicals; Macherey&Nagel; Medac; Membra Pure; Merck Biosciences; Millipore; Mobitec; MP Biomedical; NEB; Neolab; Nunc; Peske; Promega; Qiagen; Roche; Roth; Santa Cruz; Sarstedt; Semadeni; Serva; Sigma-Aldrich; Stratagene; VWR
Enzymes & Proteins & Standards	
Calf Intestine Alkaline Phosphatase	Fermentas
DNase-free RNase	Roche
DNA Standards	Fermentas
Glycogen	Roche
Lysozyme	VWR
Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase	Fermentas
Phusion Flash® High Fidelity	Finnzymes
Protein Standards	Fermentas
Proteinase K	Sigma-Aldrich
Pronase	Sigma-Aldrich
Restriction Endonucleases	Fermentas; New England BioLabs
RNasin® Plus RNase Inhibitor	Promega
T4 DNA Ligase	Fermentas
T4 DNA Polymerase	Fermentas

Taq Polymerase	Fermentas; Axon
TEV protease	This study
Vent$_R$® DNA polymerase	New England BioLabs
Zymolase 20T	Medac
Zymolase 100T	Medac
Antibiotics & Drugs	
6-Azauracil	Sigma-Aldrich
Ampicillin	Roth
Chloramphenicol	Sigma-Aldrich
Chloronaphtol	Sigma-Aldrich
Clasto-Lactacystin	Boston Biochemicals; Stratagene
Cycloheximide	Sigma-Aldrich
Geneticin	Serva
Kanamycin	Roth
MG-132 proteasomal inhibitor	Biomol
MycoPhenolic Acid (MPA)	Sigma-Aldrich
N-ethylmaleimide (NEM)	Sigma-Aldrich
Commercially available kits	
ECL kit	Applichem
DC Protein Assay kit	Biorad
Nucleobond AX PC100	Macherey & Nagel
Nucleospin Mini	Macherey & Nagel
Nucleospin extract	Macherey & Nagel
RNeasy MinElute Cleanup Kit	Qiagen
West Dura ECL kit	Thermo

4.6. GROWTH MEDIA & GENERAL BUFFERS

Table 9 | Media and buffers used in this study.

Name	Description
Growth media	
LB (Luria-Bertani Broth)	1% (w/v) tryptone; 0.5% (w/v) yeast extract; 0.5% (w/v) NaCl (+2%(w/v) agar for selective media plates)
Yeast Full Media (YPD or YPG)	2% (w/v) peptone; 2% (w/v) glucose (or galactose); 1% (w/v) yeast extract (+2% (w/v) agar for selective media plates)
Synthetic Dextrose Complete (SDC)	0.67% (w/v) yeast nitrogen base; 0.06% (w/v) complete synthetic mix of amino acids; drop out as required; 2% (w/v) glucose; when required 0.1% (w/v) 5'-FOA was added; (+2% (w/v) agar for selective media plates)
Synthetic Galactose Complete (SGC)	0.67% (w/v) yeast nitrogen base; 0.06% (w/v) complete synthetic mix of amino acids; drop out as required; 2% (w/v) galactose; when required 0.1% (w/v) 5'-FOA was added; (+2% (w/v) agar for selective media plates)
Sporulation Media (YPA)	1%(w/v) yeast extract; 2% (w/v) peptone; 1% (w/v) potassium acetate; (+2% (w/v) agar for selective media plates)
General Buffers, dyes & solutions	
4x Stacking gel buffer	0.5M Tris; 0.4% (w/v) SDS; pH 6.8 at 25°C
4x Separation gel buffer	3 M Tris; 0.4% (w/v) SDS; pH 8.8 at 25°C
10x Tris glycine electrophoresis buffer	250 mM Tris; 1% (w/v) SDS; 1.9 mM glycine
4x Sample loading buffer	0.2 M Tris pH 6.8 at 25°C; 40% (v/v) glycerol; 8% (w/v) SDS; few grains Bromophenol Blue; 0.1M DTT

Coomassie staining solution	0.25% (w/v) Coomassie Brilliant Blue R-250; 30% (v/v) Ethanol; 10% (v/v) acetic acid
Coomassie destaining solution	30% (v/v) Ethanol; 10% (v/v) acetic acid
AmidoBlack staining solution	0.2% AmidoBlack; 10% methanol; 2% acetic acid
AmidoBlack destaining solution	90% methanol; 3% acetic acid
Ponceau staining solution	0.1% (w/v) PonceauS; 5% acetic acid
1x Wet transfer buffer	25mM Tris; 192mM glycine; 10% methanol
1x Semi-Dry transfer buffer	48mM Tris; 38mM glycine; 1.28mM SDS; 10% methanol
10x Phosphate-buffered saline (PBS)	1.37M NaCl; 27mM KCl; 20mM KH_2PO_4; 10mM $Na_2HPO_4 \cdot 2 H_2O$; when required 0.1% (w/v) Tween-20® was added
10x Tris-buffered saline (TBS)	1.37M NaCl; 27mM KCl; 125mM Tris-HCl pH 7.4 at 25°C
50x TAE	2M Tris; 100mM EDTA pH 8.0; 1M Acetic Acid
6x DNA loading dye	40% (w/v) sucrose; 0.25% Bromophenol Blue; 0.25% Xylene cyanol FF
TBE	90mM Tris-borate; 2mM EDTA
TE	1mM EDTA; 10mM Tris-HCl pH 8.0 at 25°C
100x Protease Inhibitors	0.028 mg/ml Leupeptin; 0.137 mg/ml Pepstatin A; 0,017 mg/ml PMSF; 0.33 mg/ml benzamidine; in 100% EtOH p.a.

5. METHODS

5.1. MOLECULAR BIOLOGY METHODS

5.1.1. Agarose gel electrophoresis

The method is described in detail in (Voytas 2001). In this study it was performed in 1% (w/v) agarose gels containing 0.2µg/mL ethidium bromide, with 1xTAE as electrophoresis buffer. DNA standard (200ng) was used for determining the length of the fragments.

5.1.2. Molecular cloning

Cloning procedures such as digestion of DNA fragments with restriction endonucleases, dephosphorylation and ligation of DNA fragments and transformation of newly generated vectors in *Escherichia coli* were done according to Sambrook & Russell, 2001. Commercially available kits for purification of DNA fragments from agarose gels and for preparation of plasmid DNA were used according to manufacturer's instructions.

5.1.3. Polymerase Chain Reaction (PCR)

5.1.3.1. Amplification of yeast genes or TAP-tags

Reactions were done in 50-100 µl final volume using 0.5 µM primer1, 0.5 µM primer2, 0.2mM of each dNTP, 1x KNOP buffer (50mM Tris-HCl pH 9.2, 16mM $(NH4)_2SO_4$, 2.25mM $MgCl_2$), 0.3 µl genomic DNA or 1 µl 1:20 diluted plasmid Midiprep and 1µl KNOP mix polymerase (2U *Taq*, 0.56 U Vent).

In general, the following amplification protocol was used: 2 min 94°C, [1 min 94°C, 30 s at the respective °C, 1 min / 1000 bp 68°C] for 35 cycles and 10 min 68°C.

5.1.3.2. Yeast colony PCR

Reactions were done in 25 µl final volume using 1 µM primer1, 1 µM primer2, 62.5µM of each dNTP, 750µM MgCl2 and 1x Taq buffer (Fermentas). Freshly growing yeast cells were picked with a yellow tip and added to the reaction. The reaction was boiled for 15min, before 1.5U *Taq* polymerase were added. Amplification was then performed using the following protocol: [30sec 95°C, 30sec 45°C, 60sec 72°C] for 25 cycles and 2 min 72°C.

5.1.3.3. Proof-reading PCR for cloning

Point mutations were inserted by quick change mutagenesis using Pfusion Flash® High fidelity polymerase (Finnzymes). Reactions were performed according to the provided protocol. The PCR product was then digested with 10U *Dpn*I for 2 hours at 37°C. Then, 50µl of the reaction were transformed into *E. coli* DH5αPoint mutations or plasmids were sent for sequencing to Eurofins MWG-Operon in a concentration of 150ng/µL.

5.1.4. Extraction and Ethanol Precipitation of DNA

Digested DNA fragments from plasmids or PCR products were brought to 100µL final volume. Then, 60 µl of phenol:chloroform:isoamylalcohol (25:24:1) were added to the DNA solution. After 10 min centrifugation at 16000g, the upper phase was removed, mixed with an equal volume of chloroform and centrifuged for 5min at 16000g. The upper phase was then removed, mixed with 1/10 of the volume of 3M sodium acetate pH 5.2 as well as 2 volumes of 100% ethanol. After incubation for 1 h at -20°C, the DNA was precipitated and washed with 70% ethanol. The pellet was dried and resuspended in 10µl of TE buffer and used for subsequent reactions.

5.2. YEAST SPECIFIC METHODS

5.2.1. Cell density of a yeast culture

The cell density of a yeast culture was determined in a spectrophotometer at a wavelength of 600nm. One Optical Density at 600 nm (1 OD_{600}) corresponds to $2.5x10^7$ cells.

5.2.2. Transformation of yeast cells

For the transformation 50 ml of yeast were grown to an OD_{600} of 0.5 to 0.8 and harvested by centrifugation for 3 min at 3600rpm using a Rotanda 46R centrifuge. After washing with 10 ml H2O, the pellet was resuspended in 500µl of Solution I. After centrifugation, the pellet was resuspended in 250µl Solution I. Every reaction consisted of 1µl of Midi-prep DNA or 5µl of Mini-Prep DNA, 5µl of single strand carrier DNA (DNA of salmon or herring testis, 2mg/ml), 50µl of cells in Solution I and 300µl of Solution. The reaction was incubated for 30min on a turning wheel at 25°C. The transformations were then heat-shocked for 10min at 42°C, followed by 3min incubation on ice. Then, 1ml of ddH_2O was added and the cells were pelleted by centrifugation. The cell pellet was resuspended in 50µl H_2O and plated on drop-out selective plates according to the selection marker of the

transformed DNA fragment. For genomic integrations, that required homologous recombination to take place, the cells were allowed to recover. Specifically, the cell pellets were resuspended in 1ml of full yeast media and incubated for at least 1h at 25°C on a turning wheel prior to plating. To transform yeast cells grown on plate, 1 loop of freshly restreaked yeast cells was resuspended in 100mM Li-acetate and mixed well. The transformation reaction was prepared as described above. After the 30min incubation at 25°C, 35µl of DMSO were added prior to heat shock. Afterwards the reactions were treated as described above.

Solution I	Solution II
10 mM Tris-HCl pH 7.5	10 mM Tris-HCl pH 7.5
1 mM EDTA	1 mM EDTA
100 mM Li-acetate	100 mM Li-acetate
	40% PEG-4000

5.2.3. Preparation of yeast genomic DNA

For this purpose, 10ml of an overnight saturated yeast culture with an OD_{600}>1 were centrifuged (3min, 3600rpm) and washed with 10ml H_2O. The cells were resuspended with 500µl H_2O. To the cell suspension 200µl Lysis buffer, 300µl glass beads and 300µl phenol:chloroform:isoamylalcohol (25:24:1) were added. The mixture was then vortexed at full speed for 3min. After centrifugation for 10min at 16000g the upper phase was removed and extracted with an equal volume of chloroform. Genomic DNA was precipitated by addition of 1.2ml of 100% ethanol and incubation of the solution for 10min at -20°C. After centrifugation for 30min at 4°C and 16000g, the pellet was dried and resuspended in 400µl TE . To degrade the RNA, 20µl RNaseA (10mg/ml) were added and incubated for 40min at 37°C. Genomic DNA was then precipitated by addition of 40µl 3M Na-acetate pH 5.2 and 800µl of 100% ethanol and incubation of the mixture for 10min at -20°C. After centrifugation (16000g, 4°C, 30min) the pellet was washed with 80% ethanol, dried and resuspended in 30µl TE.

Lysis Buffer
2% Triton X-100
1% SDS
100mM NaCl
10mM Tris-HCl pH 8.0
1mM EDTA

5.2.4. Dot spot test

Dot spot test was used to assay the growth of yeast clones on different conditions *e.g.* selective media; drugs and temperatures. For this, 1 loop of freshly growing cells was resuspended in 1ml water. After performing five 10-fold serial dilutions, 10-15µl of the cell suspensions were spotted onto the corresponding plate (YPD, SDC-X with or without the addition of drugs). Plates were then incubated upside down to the desired temperature.

5.2.5. Epitope tagging of proteins

In yeast, tagging of genes by chromosomal integration of PCR-amplified cassettes is a standard method to label proteins *in vivo*. This 'one-step tagging' strategy directs the amplified tags to the desired chromosomal loci due to flanking homologous sequences provided by the PCR-primers. These tags are combined with different selectable marker genes, resulting in PCR amplificable cassettes. For the genomic integration of a Tandem Affinity Purification (TAP) tag to the N- or the C-terminus of the targeted protein, the method described in (Puig, Caspary et al. 2001) was essentially followed. The created PCR-product was extracted and ethanol precipitated. The purified DNA was then transformed into the yeast cells to achieve integration into the genome by homologous recombination. Transformants were tested for the presence of the tag by western Blotting.

5.2.6. Single gene deletions

In order to disrupt a gene, its coding sequence has to be replaced by an auxotrophy marker. This is achieved by homologous recombination using a construct carrying overlapping regions in the promoter and terminator region of the respective gene, separated by an auxotrophy marker. The geneX::MARKER construct was cloned by PCR amplifications of a 500bp RestrictionEnzyme(RE)I-BamHI promoter and a 300bp BamHI-REII terminator fragment of GeneX, which were ligated into the REI-REII sites of the pBluescriptIIKS(+) plasmid resulting in pBSKS-5'-*ΔgeneX*-3'. The BamHI MARKER fragment from the YDp series of plasmids (Berben, Dumont et al. 1991) was then inserted into the BamHI site of pBS-5'-*ΔgeneX*-3'. The GeneX shuffle strain was generated by transformation of the REI-REII geneX::MARKER fragment in a diploid W303 strain and selection for MARKER$^+$ transformants. Deletion of geneX was assessed by colony PCR using primers that anneal in the promoter of geneX and in the MARKER gene sequence. Positive heterozygous transformants were transformed with pRS316-GENEX. Cells were sporulated and tetrads dissected using a tetrad dissection microscope. Correct deletion process was confirmed by a

2:2 ratio of two spores growing and two spores dying on SDC-Marker plates. Haploid geneX::MARKER spores were selected for further experiments.

5.2.7. Mating of yeast strains

In order to combine two or more gene deletions, the single deletion strains were mated. For mating, haploid parental strains carrying opposite mating types were mixed onto YPD plates. After several hours, the characteristic diploid cells were selected using a dissection microscope onto YPD plates. The next day, the grown colonies –derived from the single diploids- were restreaked on the necessary plates.

5.2.8. Sporulation and tetrad dissection

For sporulating a diploid strain, freshly grown diploid cells were restreaked onto sporulation plates. On these plates, the diploid cells undergo meiosis and the genetic information is divided in four haploid spores, enclosed in a tetrad. Sporulation was monitored on a light microscope. The outer cell wall of the tetrad was destroyed by incubating 1 loop of cells in 10µl Zymolase20T. The destruction was stopped by addition of 30µl of 1xTE, and the spores could be dissected using the tetrad microscope. Tetrads with four growing spores were then restreaked onto YPD and the respective drop out plates to check for segregation of markers. Double knock out strains were selected by checking for the correct 2:2 auxotrophy marker distribution.

5.2.9. Depletion of genes by glucose repression

A genomic depletion system for the targeted geneX was designed based on a strain carrying a C-terminal TAP-tagged version of geneX (allowing determination of protein levels using the PAP antibody) driven by the GAL1 promoter (GAL1::geneX-TAP). Cells were grown in galactose-containing media (YPG, GeneX expressed) to mid-log phase. Cells were then centrifuged (3min, 3600rpm), washed with H_2O and resuspended in glucose containing medium (YPD), where the expression of geneX was repressed. A small amount of cells (5 OD_{600}) was taken every 2h to monitor by western blot for the reduction in the protein levels of ProteinX.

5.2.10. Growth curve analysis

In order to analyse the growth of different yeast strains, a growth curve analysis was performed. For this, the optical density of three independent yeast cultures was assessed

every hour and documented. The cultures were always kept in mid-log phase and so were diluted to an OD_{600} of 0.3 as soon as they reached an $OD_{600} \geq 1$.

5.2.11. Yeast Whole Cell Extracts (WCE)

5.2.11.1. Glass beads preparation

For analytical WCEs a 2ml culture was inoculated with one loop of freshly grown cells and grown over night to saturation. Cells were harvested (3min, 3600rpm), resuspended with 95°C hot 100µl 1x Sample Buffer (1xSB) and vortexed with 100µl glass beads as following: 3 x [1min vortexing, 3 min 96°C]. After 5min spin at 16000g, the supernatant was isolated from the glass beads and was used for further experiments. For native WCEs, cells were grown to mid-log phase and 10 OD_{600} were harvested and washed with 1ml 1x TAP buffer (for recipe see below). The pellet was resuspended in an equal volume of 1x TAP buffer and lysed with the double volume of glass beads (4x2min vortex, 2min ice). After a low spin (3min, 1500g, 4°C), the supernatant was centrifuged for 30min at 16000g and 4°C. The protein concentration was determined using the DC Protein Assay kit.

5.2.11.2. Denaturing protein extraction

Denaturing protein extraction from yeast cells was carried out essentially as described (Knop, Finger et al. 1996). Briefly 5 OD_{600} of cells were resuspended in 500µl of ddH_2O, 150µl of pre-treatment solution were added and the mixture was incubated for 20min on ice. After addition of 150µl of 55% (v/v) Trichloroacetic acid (TCA) and a further 20min incubation on ice, the tubes were centrifuged for 20min at 16000g and 4°C. The supernatant was removed and the pellets were resuspended with 100µl of 1xSB and 20µl of 1M Tris base. The samples were then incubated for 2min at 95°C. After 5min centrifugation at full speed the supernatant was ready for use in further experiments.

Pre-treatment solution

7.5% (v/v) βmercaptoethanol

1.85M NaOH

5.2.12. Long-term storage of yeast cultures

To store yeast cultures for longer periods, freshly growing cells (approximately 100µL) were resuspended in 1mL of 50% (v/v) sterile glycerol. Cell suspensions were quick freeze in liquid N_2 and stored at -80°C.

5.3. SDS-PAGE & WESTERN BLOTTING

5.3.1. SDS-PAGE

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli using the Mini-Protean II system (Biorad). Routinely, 6% to 15% polyacrylamide mini gels were used. For separation of purified complexes occasionally pre-cast 4-12% Bis-Tris gradient gels were used. After electrophoresis, separated proteins were either transferred to a membrane (western blot) or directly stained with Coomassie.

5.3.2. Western Blotting

5.3.2.1. Protein transfer

In order to detect a specific protein in a given sample of an extract, proteins were separated by SDS-PAGE and then transferred to a nitrocellulose of a PVDF membrane. Briefly the stacking gel was removed after the run and the gel was equilibrated for 1min in 1x Semidry or Wet blotting buffer. The PVDF membrane was previously activated (in methanol) and washed with 1xSemidry or Wet blotting buffer. A sandwich consisting of (from bottom to top) three layers of Whatman®-paper soaked in 1x blotting bufer, nitrocellulose or PVDF membrane, polyacrylamide gel and another three soaked Whatman®-papers was assembled in a Semidry or Wet blotting device. Semidry devices were mostly used for transfer of low molecular weight proteins onto nitrocellulose membranes while Wet blotting devices were used for transfer of high molecular weight proteins onto PVDF membranes. Proteins were transferred either at 7V for 45min (Semidry) or at 100V for 1h (Wet). Successful transfer was monitored by PonceauS or AmidoBlack reversible staining of the membrane.

5.3.2.2. Protein detection

After transfer, the membrane was blocked with blocking buffer (see below) for at least 1h. The blot was then incubated overnight at 4°C with the primary antibody dissolved in blocking buffer. Excess of primary antibody was removed by washing the membrane 3 times for 15min with blocking buffer at 25°C. The membrane was incubated with secondary antibody diluted in blocking buffer for 2h at 25°C. Signals were detected using a chemiluminescence kit, followed either by exposure of the membrane to either light-sensitive films and subsequent developing using a Kodak X omat M35 developing machine, or by exposure to a fluorescent image reader. In the latter case the images were

acquired using the Fujifilm Mini-LAS300 System (Fujifilm Life Sciences) and quantified using the MultiGauge ScienceLab2005Ver3 (Fujifilm Life Sciences).

Blocking buffers

2% (w/v) milk in 1x PBS

5% (w/v) milk in 1x TBS-Tween20

2% (w/v) BSA in 1x TBS-Tween20

5.4. TANDEM AFFINITY PURIFICATION (TAP)

TAP purification allows a rapid and clean purification of native protein complexes using a combination of two different tags, Protein A and calmodulin binding protein (CBP), separated by a TEV-cleavage site.

1x TAP Buffer	1x Elution Buffer
100mM NaCl	10mM Tris-HCl pH 8.0
50mM Tris-HCl pH 7.5	5mM EGTA
1.5mM MgCl2	
0.15% NP-40	

5.4.1. Cell harvest and lysis

For purification of native protein complexes of *S. cerevisiae* (Puig, Caspary et al. 2001), a 2 l culture of an OD_{600} of 3,5 was harvested (2min, 5000rpm). Cells were washed with ddH_2O, followed by a second washing step with 25ml 1xTAP buffer supplemented with 1xProteaseInhibitor (1xPI) cocktail (for recipe see below). Cells were then frozen in liquid N_2. An equal volume of 1xTAP buffer (also containing 1 mM DTT) and a double volume of glass beads were mixed with the cells in a bead mill, and lysed by the following milling protocol: 3x [4min, 500rpm, 2min break]. The glass beads were removed and washed once with 1xTAP buffer, so that the final volume of the lysate was 25ml. After centrifugation for 10min at 4°C and 4000rpm, the supernatant was subjected to ultracentrifugation for 1h at 100000g and 4°C using an SW32 rotor. The top fatty phase was removed by aspiration and the clear lysate was collected. For storage, 5% (v/v) glycerol was added to the lysate and the lysate was frozen in liquid N_2.

1xProteaseInhibitor (1xPI) cocktail

0.028 mg/ml Leupeptin

0.137 mg/ml Pepstatin A

0,017 mg/ml PMSF

0.33 mg/ml benzamidine

Dissolved in 100% EtOH p.a.

5.4.2. Purification and TCA precipitation

For purification, 0.4ml IgG-sepharose were washed 3x in 1xTAP buffer (2min, 1800rpm, 4°C) and were then added to the lysate. After incubation for 1h at 4°C, the beads were centrifuged down and transferred to a mobicol column, containing a 35µm filter. The beads were washed by gravity flow with 10ml 1xTAP buffer containing 0.5mM DTT. To cleave off the protein complex from the IgG beads, 6µl of TEV protease and 150µl 1xTAP buffer plus 0.5 mM DTT were added to the mobicol and incubated for 1h and 20min at 19°C. For elution, the column was centrifuged for 1min at 2000rpm. During the TEV cleavage, 0.5ml of calmodulin beads were washed 3x with 1xTAP buffer containing 1mM DTT and 4mM $CaCl_2$. After removal of surplus buffer, the beads were incubated with 150µl 1xTAP buffer containing 1 mM DTT and 2 mM $CaCl_2$ on ice. For calmodulin binding, the 150µl TEV eluate were added to the activated calmodulin beads and incubated for 1h at 4°C. The reaction was then washed with 7.5ml of 1xTAP buffer plus 1mM DTT and 2mM $CaCl_2$. To elute the protein complex, the beads were incubated in a thermomixer at 37°C for 2x; each 7.5min in 1xElution Buffer. The eluate was obtained by centrifugation for 1min at 2000rpm. To concentrate the samples, TCA was added to a final concentration of 10% (v/v) and the samples were incubated for 20min on ice. After 20 min centrifugation at 16000g and 4°C, the pellet was resuspended in 60µl 1xSB. The resulting solution was neutralized by the addition of 1M Tris-Base, denatured and subjected to SDS-PAGE.

5.5. CHROMATIN IMMUNOPRECIPITATION (CHIP)

For analyzing the occupancy of RNApolII and the proteasome on the genes, ChIP was performed essentially as described (Kuras and Struhl 1999; Strasser, Masuda et al. 2002).

1x FA lysis buffer
50mM HEPES-KOH pH to 7.5
150mM NaCl
1mM EDTA
1% Triton X-100
0.1% sodium deoxycholate
0.1% SDS

1x FA (high NaCl) lysis buffer
50mM HEPES-KOH pH to 7.5
0.5M NaCl
1mM EDTA
1% Triton X-100
0.1% sodium deoxycholate

1xTLEND
10mM Tris-HCl pH 8.0
0.25M LiCl
1mM EDTA
0.5% Nonidet P-40
0.5% SDS

ChIP elution buffer
50 mM Tris·Cl pH 7.5
10 mM EDTA

5.5.1. Cell preparation and lysis

An over night saturated culture was diluted to 0.2 OD_{600}. The culture was grown at 30°C and 120rpm until it reached mid-log phase (OD_{600} 0.7-0.8). Protein crosslinking was achieved by the addition of 1% (v/v) formaldehyde to the culture and slowly shaking at room temperature for 15min. The crosslinking reaction was stopped by addition of 375mM Glycine and further incubation for 20min. Cells were harvested by centrifugation (4000g, 5min, 4°C) and washed 2x with cold 1xTBS and 1x with 1xFA lysis buffer supplemented with 1xPI cocktail. Cell pellets were frozen in liquid N_2 and stored at -80°C. For lysis, the crosslinked cells were resuspended in 1ml of 1xFA lysis buffer and mixed with an equal volume of glass beads. The mixture was vortexed at full speed for 6x [3min vortex, 3min on ice]. The lysate was sonicated in a BioruptorTM UCD-200 (Diagenode) using 25x30 sec cycles with 30sec breaks at an output of 200W to produce chromatin fragments of average size 200bp. Cell debris were removed with a 16000g centrifugation at 4°C. Protein concentration was assessed by measuring absorbance of the lysate at 280nm (A_{280}).

5.5.2. Immunoprecipitation, elution, protein degradation and DNA purification

For precipitation, $3A_{280}$ units (for RNApolII) or $18A_{280}$ units (for Proteasome) were incubated with 15µL IgG-coated Dynabeads M280 in a total volume of 1.2mL 1xFA lysis buffer for 3.5h at 20 °C or O/N at 4°C, respectively. After immunoprecipitation, the samples were washed as previously described (Kuras and Struhl, 1999).

Immunoprecipitated material was eluted from the beads with 1xChIP elution buffer by incubating the samples for 20min at 65 °C and reversal of the crosslink was achieved by incubation with 400µg pronase O/N at 65°C. The DNA was purified using the Nucleospin extract II (Macherey & Nagel) kit with a final elution volume of 50µL. Immunoprecipitated DNA was subjected to PCR analysis.

5.5.3. Amplification of precipitated DNA by PCR

For determining the amount of the purified protein in a specific genomic region, the precipitated chromatin was subjected to quantitative Real-Time PCR (qPCR) with primers directed against specific genomic regions. Briefly, qPCR was performed on an ABI Prism 7000 machine (Applied Biosystems). The PCR reactions were carried out in 20µL containing 1xPolymerase buffer (Axon), 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.5µM each primer, 1:50.000 diluted SYBR Green I Nucleic Acid dye (Roche) and 1U of Taq polymerase (Axon). Relative quantification using a standard curve method was performed and the occupancy level for a specific fragment was defined as the ratio of immunoprecipitated DNA over total DNA. The NTR ChrV 174137-174447 region was used as a negative control. Primers used are listed in Table 6.

5.5.4. Coupling of beads

Coupling of Tosyl-Activated Dynabeads M280® (Invitrogen) to IgG antibody was done according to the manufacturer's protocol.

5.6. ANALYSIS OF RNA POLYMERASE II UBIQUITYLATION

For assessing the ubiquitylation of RNApolII, strains that carried a TAP-tagged version of Rpb3 were used to purify RNApolII. Briefly, 100 OD_{600} of logarithmically grown cells were pelleted and resuspended in 1xLysis Buffer. Cells were lysed in an equal volume of glass beads by vortexing at full speed for 5x [3min vortex, 3min on ice]. After centrifugation for 30min at 16000g and 4°C, the clear lysate was collected for immunoprecipitation. For each reaction, 50µL of IgG slurry (pre washed 3x with 1xLysis buffer) was incubated with the lysate for 2h at 4°C. The samples were then washed at least 4x with 1xLysis Buffer and proteins bound were eluted by addition of equal volume of 2X

SB and incubation for 3min at 65 ºC. The precipitated proteins were subjected to western blotting and blots were probed with anti-ubiquitin (see Table 7) antibodies.

1x Lysis Buffer

100mM NaCl
50 mM Tris-HCl pH 7.5
1.5 mM MgCl2
0.15% NP-40
2mM N-ethylmaleimide
20µM lactocystine
1x PI cocktail

5.7. QUANTIFICATION OF mRNA LEVELS

5.7.1. RNA extraction

RNA was extracted from yeast as previously described (Köhrer and Domdey, 1991).

5.7.2. cDNA synthesis and qPCR

The extracted RNA was subjected to first strand cDNA synthesis using oligo-dT as primer and the M-MuLV Reverse Transcriptase (Fermentas). Reaction was carried out according to the Fermentas instructions. The cDNA was then analyzed by Real-Time PCR on an ABI Prism 7000 machine (Applied Biosystems). Reactions were performed as mentioned above for the ChIP. Relative quantification using a standard curve method was performed and the relative mRNA level of Rpb1 or Rpb3 was defined as the ratio of the Rpb1 or Rpb3 mRNA over the Adh1 or Act1 mRNA. Primers used are listed in Table 6.

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7. ABBREVIATIONS

6AU	6-Azauracil
Å	Angstrom
ATP	adenosine triphosphate
bp	basepair
°C	degree centigrade
ChIP	Chromatin Immuno Precipitation
CTD	C-Terminal Domain of the largest RNAPII subunit
d	day
Da	dalton
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleosid triphosphate
E. coli	Escherichia coli
ECL	enhanced chemoluminiscence
et al.	et alli (Latin and others)
5'-FOA	5-Fluoorotic Acid
g	gram
GGR	Global Genome Repair
h	hours
kDa	Kilo Dalton
L	litre
LB	Luria-Bertani
M	molar
min	minute
MDa	Mega Dalton
mRNA	messenger RNA
NER	Nucleotide Excision Repair
nt	nucleotide
OD	optical density
ORF	Open Reading Frame
PAGE	polyacrylamide gelelectrophoresis
PBS	Phosphate-buffered saline
PBST	PBS with 0.1 % Tween 20
PCR	Polymerase Chain Reaction
pH	potential of hydrogen
RNA	ribonucleic acid
RNAP	RNA Polymerase
qPCR	quantitative PCR
rpm	rotation per minute
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	Reverse Transcription PCR
S	Svedberg
SDS	Sodium Dodecyl-Sulphate
SL	Synthetic Lethal
snoRNP	small nucleolar RNP
TAP	Tandem Affinity Purification
TBS	Tris-buffered saline
TBST	TBS with 0.1 % Tween 20
TCA	Trichloroacetic Acid
TCR	Transcription Coupled Repair
TEV	Tobacco Etch Virus
TREX	Transcription/Export complex
tRNA	transfer RNA
ts	temperature sensitive
UPP	Ubiquitin Proteasome Pathway
µ	micro

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Family status	Single

EDUCATION

2005-Present **PhD student in Biochemistry**
Lab of Dr. Katja Straesser , Gene Center Munich, Germany
Thesis title: Molecular mechanism for degradation of transcriptionally stalled RNA polymerase II in the yeast *Saccharomyces cerevisiae*

2004-2005 **Diploma Thesis in Immunology**
Lab of Prof. Dr. George Mosialos, "Biomedical Sciences Research Center (B.S.R.C) Alexander Fleming", Greece
Thesis title: Suppression of Epstein-Barr virus Latent Membrane Protein-1 (LMP-1) by RNA-Interference

2000-2005 **Diploma in Molecular Biology and Genetics**
Democritus University of Thrace, Greece
Grade 'Excellent', (8.62 at best 10)

2000 **Panhellenic Exams for Entrance in Higher Education**

1997-2000 **Highschool (Lyzeum) Diploma**
2nd Unified Highschool, Glyfada, Greece

1994-1997 **Junior Highschool (Gymnaseum) Diploma**
2nd Unified Highschool, Glyfada, Greece

1988-1994 **Elementary school**
Marasleios Normative Academy

AWARDS AND FELLOWSHIPS

2006-2009 **PhD Fellowship**
Boehringer Ingelheim Fonds

30.03.2005 **Honorary Diploma Graduation**
Declamation of Scientific Oath at Inauguration Day

1994-2000 **Excellent Progress prizes**
2nd Unified Highschool, Glyfada, Greece