Genetic and genomic analysis of RNA polymerase II backtracking in *Saccharomyces cerevisiae*

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1. INTRODUCTION

1.1 Transcription by RNA polymerase II

Transcription is a cellular process by which an RNA molecule is synthesised from a DNA template. The central element of this process is the RNA polymerase, the enzyme that synthesises this RNA molecule from nucleotide triphosphates (NTPs). There are three RNA polymerases in the eukaryotic cell: RNA polymerase I, the enzyme that transcribes ribosomal RNAs (rRNAs) 25S, 18S and 5.8S, RNA polymerase II, responsible of the transcription of messenger RNAs (mRNAs) that will be translated to produce proteins, some small nuclear RNAs (snRNA) and other non-coding RNAs and finally, the RNA polymerase III that transcribes transfer RNAs (tRNAs), ribosomal RNA 5S and some snRNAs. These three polymerases present a similar structure and share some subunits while others are specific (Sentenac, 1985).

RNA polymerase II is a multiprotein complex consisting of twelve subunits (Cramer, 2004). These proteins are coded by the genes *RPB1-12*. All of them except Rpb4 and Rpb9 are essential for the viability of the cell (Woychik and Young, 1989; Woychik et al, 1991). Rpb1 and Rpb2 are the biggest and better conserved subunits. These subunits associate creating a channel known as the active site that binds an Mg^{2+} ion as co-factor in the inside. The rest of subunits associate around this channel to form the enzymatic complex. The active site is the place where the biogenesis of nascent RNA takes place. This channel contains approximately 20 base pairs (bp) of the template DNA that is being transcribed, as well as an RNA-DNA hybrid of 9 bp. The nascent RNA abandons the polymerase through an exit channel. Under the active site there is a pore for the entrance of NTPs. The core of RNA polymerase II is formed by ten subunits, presenting also a dissociable heterodimer that consists of the subunits Rpb4 and Rpb7 and that binds to the nascent mRNA nascent channel (Edwards et al, 1991; Gnatt et al, 2001).

There are numerous factors that are capable of interacting with RNA polymerase II to modulate transcription. Many of these interactions take place through a carboxi-terminal domain in Rpb1 that consists of a variable number of repeats of the heptapeptide Tyr-Ser-Pro-Thr-Ser-Pro-Ser (Prelich, 2002). This sequence is conserved in all eukaryotes but the number of repeats is variable depending on the genomic complexity of the species ranging from the 26 repeats in *Saccharomyces cerevisiae* to 52 in humans. This carboxy-terminal domain (CTD) plays a fundamental role in RNA polymerase II transcription regulation and it is susceptible of undergoing different post-translational modifications like glycosylation, proline isomerisation and serine phosphorylation (Meinhart et al, 2005). These modifications can modulate RNA polymerase II activity as well as the interactions between the CTD and a wide variety of factors

involved in transcription, that can play a role in processes as diverse as histone modification or mRNA processing. The phosphorylation state of the CTD is linked to the different phases of RNA polymerase II activity, so that in each of these steps we can detect a specific pattern of phosphorylation. The CTD is hypophosphorylated when the polymerase binds to the promoter, undergoing a first phosphorylation on serine 5 when transcription initiates. As RNA polymerase II elongates towards the 3' end of the gene, Ser5-P is gradually removed, and Ser2-P increases (Komarnitsky et al, 2000). However, it is not clear to what extent one event triggers the other. There are different factors that are capable of phosphorylate and modulate each type of RNA polymerase II phosphorylations during the transcription process (Bataille et al, 2012; Buratowski, 2009). In yeast, Ctk1 (Carboxy-Terminal domain Kinase 1) is the main kinase involved in Ser2-phosphorylation (Lee and Grennleaf, 1997), which begins to saturate around 600 nucleotides downstream from the TSS and sharply decreases around 100 nucleotides downstream from the poly(A) addition site (Mayer et al, 2010). A crossover of Ser5-P to Ser2-P is observed around 450 nucleotides downstream from the TSS. Therefore, short genes have higher levels of Ser5-P and lower levels of Ser2-P. The CTD Ser5 phosphorylation mark is erased by the Ssu72 (Suppressor of Sua72) phosphatase (Krishnamurthy et al, 2004), and possibly also by Scp1 (*Saccharomyces cerevisiae* CalPonin 1) (Yeo et al, 2003). Ssu72 is involved in many aspects of the transcription cycle: it associates with TFIIB in the PIC; it is required for the transition from Ser5 to Ser2-phosphorylation; and finally it forms part of the cleavage and polyadenylation complex that operates during transcription termination (Dichtl et al, 2002). Fcp1 has a positive role during transcription elongation (Cho et al, 1999; Mandal et al, 2002) and it is able to dephosphorylate serine 5 as well as serine 2 in humans although in yeast it shows a preference for serine 2 (Cho et al, 2001; Hausmann and Shuman, 2002; Lin et al, 2002). A third phosphatase has been described, Rtr1, that plays a role in the transition from serine 5 to serine 2 phosphorylation (Mosley et al, 2009). Serine 7 is phosphorylated at the 5' region of the genes and it is associated to the transcription of some special functional groups of RNAs such as snRNAs (Egloff et al, 2007).

The transcription process by RNA polymerase II can be divided in three steps: initiation in which the polymerase binds the promoter and recruits the factors that are necessary to start transcription; elongation, when the polymerase proceeds along the DNA template synthesising the RNA molecule and termination, when the synthesis of RNA is completed and the molecule is released from the enzyme (Svejstrup, 2004). Other processes related to transport and mRNA maturation occur coupled to these phases (Perales and Bentley, 2009).

1.1.1 Initiation

During transcription initiation, cyclin dependent kinases phosphorylate the serine 5 residues of the CTD (Hengartner et al, 1998). The first one is Cdk7 in humans or its homologue in yeast Kin28, components of the general transcription factor (GTF) TFIIH, also involved in the phosphorylation of serine 5 in 5' regions. TFIIH is a multifunctional enzyme that possesses ATP-dependent helicase (Ssl2 and Rad3) and kinase (Kin28) activities, necessary for an efficient transcription initiation (Kim et al, 2000; Svejstrup et al, 1996). This factor phosphorylates serine 5 after the assembly of the pre-initiation complex (PIC) at the promoter. The other kinase that acts during initiation is the factor Cdk8 in humans and its homologue in yeast Srb10. This protein is part of the Mediator complex that is associated to RNA polymerase II in the cells. This kinase phosphorylates serine 5 before the holoenzyme binds to the promoter to form the PIC. Until this phosphorylation is eliminated the binding to the promoter is inhibited. The first step in RNA polymerase II transcription is the assembly of the PIC that commences with the recognition of the promoter of the gene by the TATA binding protein, TBP, a subunit of TFIID. TBP as part of TFIID tends to bind to TATA-like promoters, that lack the consensus TATA box (only two or less mismatches). 80%-90% of yeast promoters fall into this category and have a PIC assembly mechanism and chromatin architecture significantly different from TATA genes (Rhee and Pugh, 2012). When TBP is not present at TFIID, SAGA directs it to TATA containing promoters. When compared to TATA-like genes, TATA-containing genes are characterized as having a propensity for being expressed at extremely high or low levels (Basehoar et al, 2004). They are generally stress-induced and highly regulated by nucleosomes and chromatin factors. In contrast, TATA-like promoters tend to be those of housekeeping genes, which are expressed at near steady levels and require less regulation (Huisinga and Pugh, 2004).

The interaction between TBP and the DNA is stabilised by TFIIA (Coleman et al, 1999) and it allows the recruitment of RNA polymerase II through TFIIB, able to interact with TBP (Conaway et al, 1991; Flores et al, 1991; Treutlein et al 2012). Next, other transcriptional factors (TFs) are recruited such as TFIIF, TFIIE and TFIIH, to conform the closed promoter complex (Boeger et al, 2005). Once all the factors have been recruited, the DNA around the transcription start site (TSS) starts to open thanks to TFIIH among others and it is inserted into the RNA polymerase II active site (Cramer 2004). This open complex is able to synthesise an RNA molecule and it will lead to a stable transcription elongation complex. Once the synthesis of mRNA commences most of the TFs dissociate from the promoter with the exception of some of them like TFIID, A and B that stay at the promoter to allow the entrance of another RNA polymerase II in a new transcription cycle (Pokholok et al, 2002; Svejstrup et al, 1997).

Additionally to all the general transcription factors, transcription initiation requires a series of factors that have the role of remodelling the chromatin and allowing the DNA to be accessible to the PIC at the promoters (Mellor, 2005). To this aim factors like Isw1, Chd1, Swr1, Rpb3, Asf1 and SAGA, SWI/SNF and FACT complexes are recruited to the promoters.

1.1.2   Elongation

1.1.2.1 Exit of the promoter

Once the open complex is formed in the promoter, occurs what is known as "promoter escape" (Dvir, 2002; Dvir et al, 2001). The CTD of RNA polymerase II is phosphorylated by TFIIH kinase and the Mediator (Kim et al, 1994; Naar et al, 2002). It is thought that this first phosphorylation produces the destabilisation of the interactions between mediator and the CTD, allowing the first to act on new initiating polymerases (Svejstrup et al, 1997). Many GFTs are disassembled. These GFTs stay at the promoter to help the formation of the next PIC, leaving space for the contact of elongation factors with the CTD (Sims et al, 2004; Zawel et al, 1995). In this step occurs the synthesis of the first phosphodiester bond of the nascent RNA, although the polymerase is not yet ready for productive transcription elongation. Firstly, short transcripts between 3 and 16 nucleotides are produced. These transcripts dissociate from the polymerase in a process denominated abortive initiation (Luse et al, 1987). Biochemical studies indicate that the threshold of stability is not reached until the addition of the fourth nucleotide (Luse et al, 1987; Kugel and Goodrich, 2002) moment at which takes place the "promoter escape commitment". This happens simultaneously to certain structural changes in RNA polymerase II that explain the threshold of stability. RNA polymerase II possesses a domain denominated clamp that interacts with the DNA/RNA hybrid and the downstream DNA template. The strength of the hold of this structure on the DNA can be modulated by five different domains of RNA polymerase II, three of which interact with the RNA/DNA hybrid, being the length of this hybrid key to diminish the strength that this clamp exerts on the downstream DNA, what facilitates the translocation of the polymerases on the template DNA (Dvir, 2002). Lastly, it has been identified an RNA exit channel in the structure of elongating RNA polymerase II that is able to accommodate single stranded nascent RNA molecules. This channel would be filled when the RNA reaches a length of 18 to 20 nucleotides. There is a narrowing at the end of this channel where the RNA-protein interactions are favoured. These interactions produce a stabilisation of the complex when the transcript reaches a length of 15-20 nucleotides. At this point the ternary complex RNA polymerase II-DNA-RNA reaches its higher stability, but it is not a mature elongation complex yet. Until the length of the transcript reaches 23 nucleotides the polymerase tends to suffer a displacement over the DNA that

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leaves the 3' end of the nascent RNA out of the active site producing a blocking of the enzyme (Pal and Luse, 2003). This situation is solved thanks to the action of the transcriptional factor TFIIS that is able to stimulate RNA polymerase II intrinsic endonucleolythic activity, that cuts the RNA leaving the free 3' end in the active site again (Cramer, 2004).

1.1.2.2 RNA polymerase II promoter-proximal pause

Once the polymerase has abandoned the promoter, before initiating productive transcription elongation it suffers a pause that acts as a check point to allow the correct assembly of transcription elongation factors and the factors that intervene in the first maturation process that the RNA undergoes: the 5' capping. It has been reported that RNA polymerase II is paused at the 5' end of almost 10% of all *Drosophila* genes (Muse et al, 2007). In mammals, most genes are also enriched with RNA polymerase II at their 5' ends (Guenther et al, 2007). Global run-on sequencing experiments confirmed that many of these promoter-proximal polymerases are indeed paused (Core et al, 2008). There have been identified two factors that play a role in this pause. The first one is DSIF, a complex consisting of the proteins Spt4 and Spt5 (Wada et al, 1998a) that is present in all eukaryotic organisms. It was identified in humans by its capacity to inhibit transcriptional elongation when the cells are treated with the kinase inhibitor DRB. The complex DSIF interacts physically with RNA polymerase II (Hartzog et al, 1998). In addition to this, Spt5 physically interacts with the enzymes that cause the maturation of the 5' end of the RNA (Lindstrom et al, 2003; Pei and Shuman, 2002). The second complex is NELF, that can be found in mammals and *D. melanogaster* but it is absent in yeast, *C. elegans* or *A. thaliana* (Narita et al, 2003). This complex increases the tendency of RNA polymerase II to pause (Yamaguchi et al, 2002) and the time of permanence in pause sites (Renner et al, 2001). NELF complex seems to be absent in yeast. Moreover, in yeast RNA polymerase II usually displays an approximately uniform distribution across the transcription unit (Steinmetz et al, 2006). Thus, the transition from transcription initiation to elongation in *Saccharomyces cerevisiae* has been suggested to be rapid, with little post-recruitment regulation (Wade and Struhl, 2008).

1.1.2.3 Productive elongation

After the initial pause of RNA polymerase II, the enzyme undergoes a series of modifications that confer it processivity, thus initiating the productive elongation step of transcription during which, unless pause or blocking situations occur, the RNA is synthesised at a constant rate. Many elongation factors interact with RNA polymerase II to allow the enzyme to carry out this process efficiently. Some of these factors confer processivity to the enzyme,

others allow it to abandon pause sites and others remodel the chromatin to allow the polymerase to proceed through a template with nucleosomes assembled (Krogan et al, 2002, Shilatifard et al, 2003). During elongation, the serine 2 residue of the CTD is phosphorylated by P-TEFb in mammalian cells, being Cdk9 the subunit that catalyses this reaction (Marshall et al, 1996; Peterlin and Price, 2006). In yeast the kinases equivalent to Cdk9 is Bur1 (Wood and Shilatifard, 2006) part of the Bur1/Bur2 complex. Another kinase that has been found in mammals is Cdk12, which equivalent in yeast is Cdk1 (Bartkowiak et al, 2010). These complexes are considered positive transcription elongation factors and are not redundant. Ctk1 mainly phosphorylates the serine 2 residues of the CTD (Cho et al, 2001). Bur1/Bur2 associates to the serine 5, phosphorylated by Kin28, near the promoter and it regulates the monoubiquitylation of H2B via Rad6 phosphorylation and the recruitment of the PAF complex. Bur1/Bur2 can phosphorylate serine 2 which favours the trimethylation of H3 in the lysine 36 (H3-K36Me3) by PAF and SET2 along the transcriptional unit during elongation. The trimethylation of H3-K36 favours the recruitment of histone deacetylases, preventing the acetylation in the 3' regions and the consequent activation of cryptic promoters inside the genes (Chiu et al, 2007; Qiu et al, 2009).

1.1.2.3.1   Factors that confer processivity to RNA polymerase II

Once the cap has been formed at the 5' end of the RNA the pause of the polymerase is abolished and transcription goes into the stage of productive elongation. The determinant factor in this transition is p-TEFb (Marshall et al, 1996; Peterlin and Price, 2006). It is able to counteract the negative effect of DSIF and NELF on transcription elongation (Wada et al, 1998b; Yamaguchi et al, 1998). P-TEFb is comprised by a cyclin-kinase pair, the T1 cyclin and the kinase dependent cyclin Cdk9, that play a dual role to allow the polymerase overcome the pause. In the first place, the cyclin phosphorylates the serine 2 residues of the CTD, which confers processivity to the RNA polymerase II (Marshall et al, 1996). Secondly, Cdk9 can phosphorylate Spt5, one of the subunits of DSIF, eliminating the negative effect of this complex (Ivanov et al, 2000; Wada et al, 1998b). In yeast these processes are carried out by the homologue complexes of p-TEFb. The first of them is formed by the Bur1 kinase and the Bur2 cyclin; the second is the CTDK complex, which consist of the subunits Ctk1, 2 and 3 (Wood and Shilatifard, 2006). These complexes are not redundant and it has been hypothesised that CTDK is the complex responsible for the phosphorylation of the CTD in physiological conditions, while Bur1/Bur2 would have Spt5 as a substrate (Keogh et al, 2003), although any of them can substitute the other one in case of mutation. In this group can also be found DSIF, that also has a positive role apart from the negative role of promoting RNA

polymerase II pausing (Wada et al, 1998b). In fact, contrary to NELF that is disassembled from the RNA polymerase II after the pause, DSIF accompanies the elongating enzyme along all the length of the transcript (Andrulis et al, 2000; Kaplan et al, 2000). Mutations in *SPT4* or *SPT5* make the cell sensitive to 6-azauracil (6AU) a drug that affects elongation by depleting the level of available nucleotides triphosphate (NTPs) in the cell. Additionally, the combination of these and other mutations on genes implicated in transcriptional elongation results in a type of synthetic lethality (Costa and Arndt, 2000; Lindstrom and Hartzog, 2001). The deletion of *SPT4* renders the cell unable to transcribe long genes with a high content in G+C (Rondon et al, 2003), it diminishes its processivity (Mason and Struhl, 2005) and it provokes the accumulation of active RNA polymerases II in the 5' end of yeast genes (Rodríguez-Gil et al, 2010).

1.1.2.3.2   RNA polymerase II pausing and backtracking

RNA polymerases can undergo pauses and blockings. These situations can be planned as part of mechanisms of regulation of the expression of certain genes, or eventual as a result of conditions in which transcription is impaired. In these situations the polymerases can be paused but active, or get blocked after suffering a backtracking over the template DNA. RNA polymerase II elongates by oscillating between forward and backward movements (Bar-Nahum et al, 2005). During mRNA elongation, RNA polymerase II can undergo transient pausing that can be induced by certain DNA sequences that favour the reverse movement of the enzyme, misincorporations of nucleotides, damage on the DNA, or hairpins.   During pauses transcription is momentarily stalled but the polymerase will eventually resume transcription. To overcome pauses, a misincorporated nucleotide must be removed from the 3' end of the RNA by RNA proofreading (Sydow and Cramer, 2009). These pauses increase the chance of backtracking (Izban and Luse, 1991). Backtracking occurs when the polymerase moves backward along the DNA template and the synthesised RNA 3' end becomes extruded from the active site. In this case the 3' end is outside of the active site and needs to be cut in order for the polymerase to proceed with transcription. Once arrested the polymerase needs the intervention of additional factors to be reactivated and resume transcription elongation (Fuensanta and Cramer, 2013). The RNA polymerase II possesses a basal endonucleolythic activity that has to be induced in situations where these blockings are frequent (Awrey et al, 1997; Cramer, 2004; Fish and Kane, 2002; Sigurdsson et al, 2010). Among the factors that allow RNA polymerases to overcome pausing and backtracking are TFIIF, ELL, the elongin, TFIIS and Ccr4.
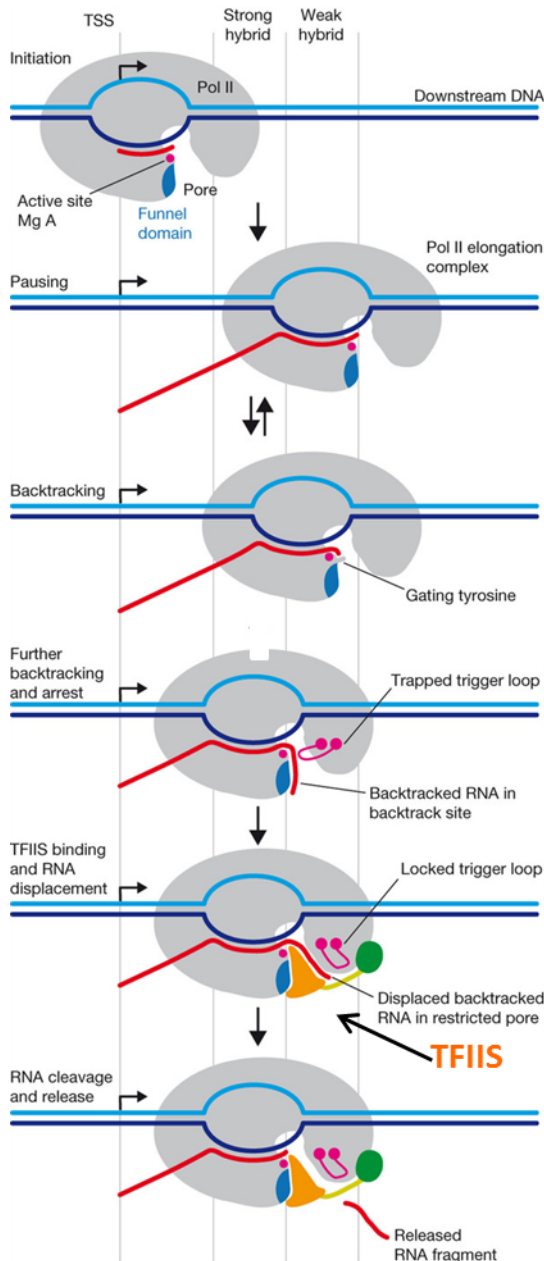
Figure 1 Schematic representation of RNA pol II pausing and backtracking and TFIIS role in reactivating backtracked polymerases. Modified from Cheung and Cramer, 2011.

1.1.2.3.2.1 Factors that decrease the time of pausing of RNA pol II

The transcription factor TFIIF, apart from its role in transcription initiation has also a role during transcription elongation stabilising the active conformation of the polymerase and decreasing the time of pausing (Bengal et al, 1991; Kephart et al, 1994; Tan et al, 1994). However the mechanism by which it exerts this function is unknown. TFIIF is found associated

to the polymerase only in the proximity of the promoters (Zawel et al, 1995) and not to active elongation complexes (Krogan et al, 2002; Pokholok et al, 2002). These data, together with the observations of Yan (Yan et al, 1999) demonstrate that TFIIF is able to reduce abortive transcription during the promoter escape phase and it indicates that the role of TFIIF during elongation is limited to its initial steps.

The ELL family of proteins consists of three members ELL1, 2 and 3 (Miller et al, 2000; Shilatifard et al, 1997a; Shilatifard et al, 1996) in mammals. There is no homologue in yeast. It has been identified a unique homologue in *D. melanogaster* (Gerber et al, 2001). It has been demonstrated in studies *in vitro* that these proteins can suppress pauses during transcription elongation (Shilatifard et al, 1997b). In Drosophila, the ELL protein colocalises with the active polymerases and it is recruited together with it to induced heat shock genes (Gerber et al, 2001). Additionally, the mutation of this factor affects mostly the transcription of long genes (Eissenberg et al, 2002).

The elongin was described for the first time in mammal cells as a complex formed by three subunits, elogin A, B and C, that are able to stimulate elongation rate *in vitro* (Bradsher et al, 1993a; Bradsher et al, 1993b). Subunit A stimulates elongation, subunit C enhances A's activity and B is a chaperone that facilitates the union of the other two subunits (Aso et al 1995). It has been described that the elongin can diminish the time of pausing of the polymerase (Moreland et al, 1998). In *S. cerevisiae* there were identified two homologues of elongin A and C denominated Ela1 and Elc1. However, although Elc1 can substitute its homologue stimulating human elongin A activity (Aso and Conrad, 1997), the complex Elc1-Ela1 is not able to stimulate RNA polymerase II *in vitro* (Koth et al, 2000).

1.1.2.3.2.2 Factors that act on backtracked RNA polymerases: TFIIS and Ccr4

TFIIS is a factor that was discovered first in humans (Natori et al, 1973) and later in yeast (Sawadogo et al, 1980). It is highly conserved in archaea and eukarya (Fish and Kane, 2002; Hausner et al, 2000). There are orthologues in bacteria but their structural nature is different (Borukhov et al, 1993; Opalka et al, 2003). TFIIS is not essential through *Saccharomyces cerevisiae* cell cycle (Nakanishi et al, 1992) although it is essential in trypanosome (Uzureau et al, 2008) and it is required for the correct embryonic development of mouse and Xenopus (Ito et al, 2006; Taira et al, 2000) or plant seeds dormancy (Grasser et al, 2009). It has the ability of forming a binary complex with the RNA polymerase II to stimulate the intrinsic nucleolytic activity in the active site region of the enzyme (Weilbaecher et al, 2003; Sigurdsson et al, 2010). TFIIS is organised in three domains defined by limited proteolysis

and RMN (Awrey et al, 1998; Awrey et al, 1997; Olmsted et al, 1998). The tridimensional structure of TFIIS and the polymerase has been determined by crystallographic studies (Kettenberger et al, 2003, 2004, Cheung and Cramer, 2011). The N-terminal protrudes from RNA polymerase II and it is not required for transcription elongation (Awrey et al, 1998, Nakanishi et al, 1995) but physically interacts with Med13 (Srb9) a component of the Cdk8 subcomplex of mediator and Spt9 of SAGA (Wery et al, 2004). The central domain inserts into the pore of the polymerase leaving the C-terminal domain and consequently the conserved RSADE motive very close to the active site where it is thought two acid residues complex an essential metallic ion to allow the endonucleolytic cleavage (Kettenberger et al, 2003). In *S. cerevisiae,* TFIIS is coded by the gene *DST1/PPR2.* The deletion of *DST1* as well as mutants where the RSADE domain has been deleted do not possess effects on cell growth except at low temperatures (Ghavi-Helm et al, 2008) or in the presence of drugs that deplete the pool of NTPs in the cell compromising the elongation process, like 6AU or MPA (Exinger and Lacroute, 1992). TFIIS is not required in normal conditions, but it becomes essential when transcription is impaired. In the presence of 6AU the deletion of *DST1* affects RNA polymerase II processivity not affecting its transcription rate (Mason and Struhl, 2005). TFIIS can stimulate transcription to overcome artificial arrests *in vivo* demonstrating that it allows the polymerase to overcome arrests (Kulish and Struhl, 2001). It has been demonstrated that the endonucleolytic cleavage of the polymerase is essential for cell viability which indicates that blocking situations in optimal growth conditions might be frequent (Sigurdsson et al, 2010). The implications of the importance of this factor *in vivo* remain to be clarified. Various publications suggest that apart from its role in elongation TFIIS possesses a function in initiation, in the formation of the PIC and the transition between initiation and elongation, that seem to be related to its N-terminal domain and its interaction with SAGA and mediator (Kim et al, 2007; Malagon et al, 2004; Prather et al, 2005b; Wery et al, 2004). This role in initiation does not depend on the RSADE domain and it does not require the induction of the endonucleolythic cleavage (Guglielmi et al, 2007; Kim et al, 2007). ChIP on chip studies have demonstrated its localisation not only on RNA polymerase II dependent genes but also on genes that are transcribed by nuclear RNA polymerases III in which it seems to play a role in the correct selection of the transcription start site. It has also been immunoprecipitated in regions of rDNA that corresponds to 35S (RNA polymerase I) though the role it could have in these regions has not been explained (Ghavi-Helm et al, 2008).

The Ccr4-Not complex is also involved in the reactivation of backtracked polymerases. It is a nine-subunit protein complex that has been implicated in almost all aspects of gene

control, including transcription initiation, mRNA decay and quality control, mRNA export, and translational repression (Miller and Reese, 2012). It has been proposed that the Ccr4-Not complex interacts with the emerging RNA transcript to favour the RNA polymerase II forward movement, promoting the resumption of elongation without RNA cleavage (Kruk et al, 2011). It has been described that Ccr4 and TFIIS work synergistically on arrested RNA polymerases stimulating transcription elongation. Ccr4 increases the recruitment of TFIIS and the cleavage of the extruded mRNA in backtracked RNA polymerases (Dutta et al, 2015).

There is a clear interaction between the factors that help the polymerase to overcome situations of pausing (Elmendorf et al, 2001). It has been demonstrated that TFIIF, ELL and the elongin inhibit the endonucleolytic cleavage of the nascent RNA induced by TFIIS. It has been proposed that these three factors help to reduce the time of pausing preventing the 3' OH end of the RNA from being out of the active site of RNA polymerase II and thus preventing the blocking of the polymerase what makes unnecessary the role of TFIIS. Many of these factors play relevant roles during occasional blockings, like for instance during the resolution of RNA polymerase blockings associated to DNA damage, and play an active role in the process of transcription coupled repairing (Mellon 2005; Svejstrup, 2003). Another example are the heat shock genes in Drosophilla, that need TFIIS because the polymerase is paused at the 5' end of the gene and it is liberated after the heat shock, allowing a fast transcription (Adelman et al, 2005). This also indicates that at least part of these paused polymerases have suffered backtracking.

1.1.2.3.3   Factors that modify chromatin during RNA polymerase II transcription elongation

In eukaryotic cells the DNA is packaged into the chromatin forming a compact nucleoprotein complex. The basic unit of the chromatin is the nucleosome, an octamer of histones that consists of an H3/H4 tetramer, two H2A/H2B dimers and it is circled by 146 base pairs of DNA (Luger et al, 1997). Chromatin contains a repeating array of nucleosomes that are spaced every 160-200 base pairs throughout the genome. The fact that the DNA is packaged into the chromatin implies a higher level of complexity for the regulation of all the processes that require an access to DNA. Although transcription in the cells occurs efficiently, the organisation of the DNA around the chromatin inhibits *in vitro* transcription at the level of both initiation and elongation (Izban and Luse, 1991; Knezetic and Luse, 1986). A nucleosome is a barrier for the transcriptional complex *in vitro* especially when pausing occurs. In this case the nucleosome can induce the blocking of RNA polymerase II stabilising its paused conformation after undergoing backtrack (Kireeva et al, 2005).

The position of nucleosomes at several model genes has been mapped in *Saccharomyces cerevisiae* by measuring the sensitivity of chromatin to micrococcal nuclease (MNase). MNase preferentially cuts the linker DNA connecting two nucleosomes (Axel, 1975), whereas nucleosomal DNA is partially protected against MNase digestion (Clark and Felsenfeld, 1971). The recent advances in genome-wide mapping technologies have provided a clearer picture of the organisation of nucleosomes around protein-coding genes. There is a predominant nucleosome located upstream of the TSS, designated as -1, which can regulate the accessibility of promoter regulatory elements. Downstream of the -1 nucleosome, there is a nucleosome free region (NFR) that is followed by the +1 nucleosome (Yuan et al, 2005). In yeast, the TSS is blocked by the +1 nucleosome, suggesting that this nucleosome can potentially regulate TSS accessibility (Albert et al, 2007). Beyond ~1 kb from the TSS there is an increasing tendency for random nucleosome positions. The array of nucleosomes that covers a gene terminates with a NFR at the 3′ end of the gene, and this marks the region at which RNA polymerase II terminates transcription. Thus, nucleosomes are not stochastically dispersed along genes, but they are positioned at specific distances from the TSS to regulate transcription. The statistical positioning model predicts that the positioning of one nucleosome in the array forces the positioning of all other nucleosomes due to the restriction of lateral movements as a result of the tight packing [74]. Therefore, a single genomic barrier can potentially position many nucleosomes. Of all the nucleosomes found in and around genes, the +1 nucleosome displays the tightest positioning and could provide the barrier for statistical positioning. The DNA sequence patterns AA, TT and TA occur at 10 base pairs intervals within well-positioned nucleosomes but sequence-based nucleosome positioning is largely restricted to promoter regions (Mavrich et al, 2008). Positioning is likely to involve a combination of these favourable positioning sequences as well as linker-enriched unfavourable sequences, such as the 5′ NFR. Chromatin remodeling complexes expand and contract the boundaries of the NFR (Zhang et al, 2011). Overall, nucleosome positioning is not determined by any single factor, but rather depends on the combined effects of several factors, including DNA sequence, DNA-binding proteins, chromatin remodelling complexes and the RNA polymerase II transcription machinery.

There are factors that act on the chromatin and allow the polymerase to proceed to complete the transcription (Svejstrup, 2002). Chromatin remodelling complexes are enzymes that hydrolyse ATP to reposition nucleosomes along a DNA template. They fall into four families based upon sequence conservation: SWI/SNF, INO80/SWR1, ISWI and CHD.

Histone chaperones are histone-binding proteins involved in the regulation of transcription-coupled changes in chromatin. They typically function in chromatin disassembly and reassembly. During transcription, the evicted histones can be transferred to histone chaperones by chromatin remodelling complexes. In addition, defects in some histone chaperones lead to histone depletion from transcribed genes, suggesting that histone chaperones may act as a sink for histones released during transcription as well as a source of histones during chromatin reassembly after RNA polymerase II passage.

1.1.2.3.4   Passing of the polymerase through the nucleosome barrier

Nucleosomes occupy the coding region of nearly all genes and have been shown to inhibit the elongation rate and processivity of RNA polymerase II *in vitro* (Izban and Luse, 1991). However, efficient maintenance of chromatin structure during passage of RNA polymerase II is important for cell survival and functioning. Two different modes have been proposed for RNA polymerase II passage through a nucleosome (Kulaeva et al, 2013).

It has been shown that histones H2A and H2B are exchanged at a much higher rate than histones H3 and H4 during moderate transcription *in vivo* (Dion et al, 2007; Jamai et al, 2007). Since histones H3 and H4 contain the majority of the sites for post-translational modifications, this mechanism is compatible with the maintenance of epigenetic marks during transcription. The mechanism of transcription of RNA polymerase II through a nucleosome *in vitro* is characterised by the displacement of a single H2A-H2B dimer (Kireeva et al, 2002). After a single round of transcription, the histone hexamer withstands RNA polymerase II passage and remains at the original position on DNA (Kulaeva et al, 2009). This matches the apparent effect of RNA polymerase II transcription *in vivo*. The histone hexamer does not leave the DNA during the progression of RNA polymerase II *in vitro* (Kulaeva et al, 2009). When RNA polymerase II approaches and enters the nucleosome, a small intranucleosomal DNA loop containing the transcribing enzyme is formed, allowing further transcription through the nucleosome (Kulaeva et al, 2009). As RNA polymerase II proceeds through the nucleosomal DNA, it encounters areas of strong DNA-histone interactions that cause nucleosome-specific pausing. Once RNA polymerase II pauses, the duration of pausing can be further modulated by the probability of RNA polymerase II backtracking (Kireeva et al, 2005) (figure 2 A).

Alternatively, all core histones can be displaced and exchanged at transcribed regions *in vivo* (Katan-Khaykovich and Struhl, 2011). This displacement of histones also occurs during transcription by multiple RNA polymerase II complexes *in vitro* (Kulaeva et al, 2010). It has been proposed that the two alternative histone dynamics depend on the intensity of

transcription (Kulaeva et al, 2013). During moderate transcription, the generated dimers re-bind to the hexasome before the next RNA polymerase II arrives. As the transcription rate increases, the distance between polymerases becomes shorter so the trailing RNA polymerase II may encounter the hexasome, forming an unstable intermediate that ultimately results in histone eviction from DNA (Kulaeva et al, 2010) (figure 2 B).
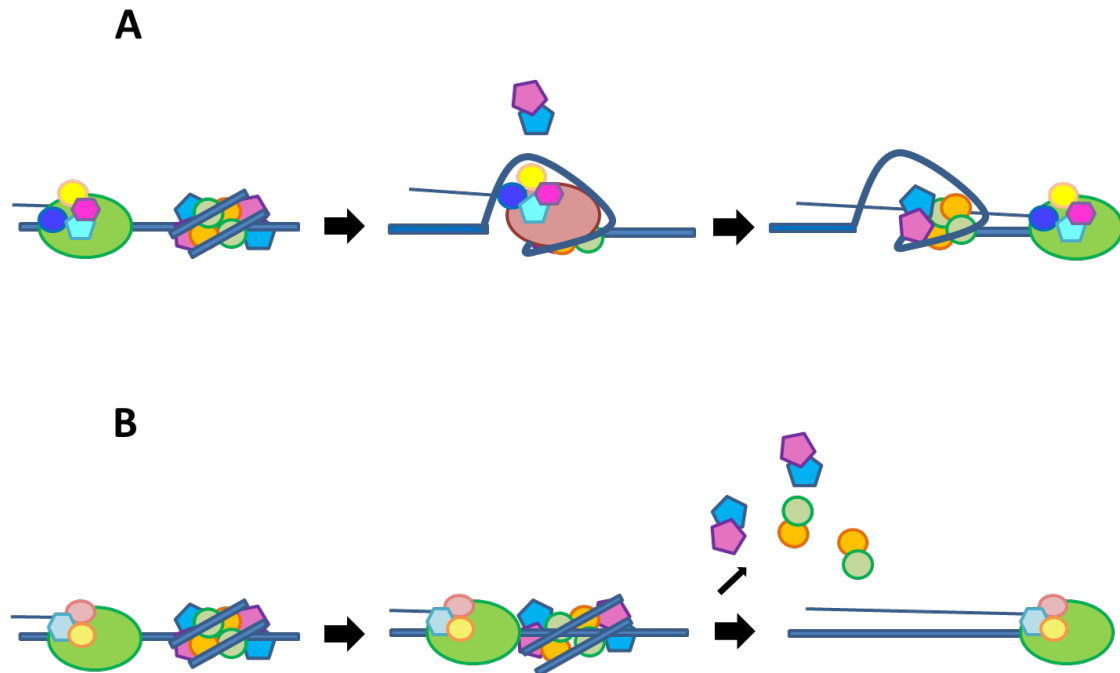

Figure 2 Model showing the two different modes of RNA polymerase II passage through a nucleosome.

1.1.3 Termination

Transcription termination is the process by which the transcribed RNA dissociates from the RNA polymerase II. This process is linked to the endonucleolythic cleavage and polyadenylation of the 3' end of the mRNA (Connelly and Manley, 1988; Logan et al, 1987). This process allows the release of the polymerase from the template and guarantees the accurate functionality of the mature mRNA. The poly (A) tail is not encoded in the DNA. A polyadenylating enzyme that forms part of the 3' end processing machinery is needed to add the tail. Several proteins that are required for 3′ end processing and transcription termination are recruited to the elongation complex by interacting with Ser2-P CTD (Richard and Manley, 2009). In fact, some of the factors that intervene in the 3' end processing of the mRNA are essential for transcription termination (Proudfoot, 2004; Proudfoot et al, 2002). These proteins include Pcf11, a component of the cleavage-

polyadenylation factor (Barilla et al, 2001) and Rtt103, a factor involved in transcription termination (Kim et al, 2004). There are two models that explain the dependency between both processes. The first one proposes that the apparition of the sequence of polyadenylation in the RNA entails a change in the factors, displacing the polyadenylation factors and giving place to positive elongation factors or else recruiting a negative factor (Connelly and Manley, 1988). In the second model, the endonucleolythic cleavage of the RNA in the polyadenylation site produces an unprotected 5' end that could lead to the entrance of an exonuclease that degrades the RNA dissociating the polymerase from the template DNA (Logan et al, 1987). Apart from the requirement of polyadenylation sequences, there are studies that indicate that termination is also facilitated by an induced pause downstream this site (Yohana and Proudfoot, 1999). A consensus sequence for this termination site does not exist. It is thought that it occurs stochastically downstream the polyadenylation site (Tran et al, 2001). In addition to poyA-coupled termination, dismantling of elongation complexes can be induced by road-block factors, like Reb1 (Colin et al, 2014). Transcription termination pathways involved in the production of non-conding RNAs, such as the Nrd1-Nab3-Sen1 pathway in yeast (Arndt and Reines, 2015), play also a role in transcription quality control and the shape of the transcriptome (Porrua and Libri, 2015). Interestingly, the exosome RNA decay complex has also been shown to participate in transcription termination in *S. pombe.* In case of backtracked RNA polymerase II, the exosome can act on the free 3' end of the nascent RNA, what results in the disassembly of the elongation complex and the degradation of the transcript (Lemay et al, 2014).

1.1.4   Processes associated to transcription

The maturation of the primary transcript of RNA polymerase II in the nucleus requires various enzymatic activities (Howe, 2002) that include the processing of the 3' and 5' ends and the elimination of introns. These processes have as substrates the pre-mRNAs synthesised by RNA polymerase II, but not the RNA transcribed by the RNA polymerases III and I or the snRNAs synthesised by the RNA polymerase II so there must be a mechanism that restricts these processes to mRNAs exclusively. For this purpose, the proteins that carry out these enzymatic activities are associated to the RNA polymerase II during transcription.

1.1.4.1   Pre-mRNA 5' end processing

The addition of the cap to the 5' end of the pre-mRNA stabilises it against the exonucleolytic degradation 5'-> 3', promotes the elimination of introns and the processing of the 3' end. It also facilitates the transport to the cytoplasm and favours translation (Lewis and

Izaurralde, 1997). This process consists of three enzymatic activities: a 5' triphosphatase that eliminates the γ phosphate of the first transcribed nucleotide, a guanililtransferase that binds a guanosine by a 5'-5' triphosphate bond, and a 7-methyltransferase that modifies the terminal guanine. In yeast the first and second activities are carried out by two distinct proteins, coded by the genes *CET1* and *CEG1* respectively that form a heterodimer, while in mammals the protein Mce1 contains both activities. As it was already mentioned in section 1.1.2.1 the pause of the polymerase helps the recruitment of processing factors to the transcriptional complex by their interaction with Spt5 (Lindstrom et al, 2003; Pei et al, 2003; Pei and Shuman, 2002) with the phosphorylated CTD (McCraken et al, 1997; Yue et al, 1997) preferentially in the serine 5 residue (Komarnitsky et al, 2000).

1.1.4.2 Intron processing

Nearly all mammalian pre-mRNAs contain introns, which are regions of DNA that are transcribed but do not ultimately form part of the mature mRNA. The process of removing these regions is called splicing. Although introns are scarce in *Saccharomyces cerevisiae* (an average of 0.05 introns per gene), splicing has been extensively studied in this organism. The CTD interacts with several proteins that function in splicing. Intron elimination is catalysed by a ribonucleoproteic macromolecular complex (RNP) denominated maturosome (Adams et al, 1996) that contains five snRNAs and up to 100 proteins (Kramer, 1996). This complex can be immunoprecipitated along with the RNA polymerase II when using antibodies against the phosphorylated CTD (Chabot et al, 1995; Vincent et al, 1996). The CTD recruits intron elimination factors and it also stimulates their action (Hirose et al, 1999).In fact, *ctk1Δ* cells, which have low levels of Ser2-P CTD, also show defects in splicing (Phatnani et al, 2004). It has been proposed that the interaction of the splicing machinery with the CTD brings together the 5' and 3' ends of the intron, thus enhancing splicing efficiency (Morris and Greenleaf, 2000). As transcription and splicing are linked, the elongation rate has an impact on splicing and vice versa (Hsin and Manley, 2012). These processes occur cotranscriptionally (Perales and Bentley, 2009).

1.1.4.3 Synthesis of the 3' end of the mRNA

The 3' end processing consists in the addition of a polyadenine tail (poly (A)) that preserves this end from degradation and it is necessary for the transport of the mRNA to the cytoplasm. The addition of this poly (A) tail involves two enzymatic reactions, the first one is the endonucleolytic cleavage of the mRNA, as it is described in the section 1.1.3 and the second would be the synthesis of the poly (A). The site of cleavage is found between two

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regions, one defined by the AAUAAA sequence and other that is characterised for being enriched in U or GU and that it is denominated downstream sequence element (DSE). The cleavage and polyadenilation specific factor (CPSF) binds to the first one and the cleavage stimulation factor (CSTF) binds to the DSE. Afterwards, the cleavage factors I and II (CFI and CFII) interact with a complex formed by CPSF and CSTF and produce the endonucleolytic cleavage of the mRNA. Right after, the poly (A)-polymerase (PAP) synthesises the tail of polyadenine with a length of 200 bases pairs in *S.cerevisiae* after the free 3' end that results from the cleavage. The poly (A) binding protein (PABP) binds the poly (A) since the beginning of its synthesis increasing the processivity of PAP (Buratowski, 2005). As it was described in 1.1.3, this process is intimately linked to transcription and it is specifically essential for termination.

1.1.4.4 Transport of the mRNA to the cytoplasm

Once the mRNA is transcribed and matured it is transported to the cytoplasm. For this purpose it associates with a series of proteins forming a ribonucleoparticle (Aguilera, 2005). The assembly of this ribonucleoparticle happens very soon after the synthesis of mRNA, even during transcription. If it is not correctly assembled the mRNA cannot be exported and it will be degraded in the nucleus. One of the key elements in this process is the THO complex, that is composed of the Tho2, Hpr1, Mtf1 and Thp2 (Chávez et al, 2000). This complex interacts with the ribonucleoparticle and directs it to the nuclear pore. Mutants on this complex have difficulties transporting mRNA to the cytoplasm and they show defects in transcriptional elongation of long genes with high contents in G+C (Chávez and Aguilera, 1997).

1.1.4.5 Rpb4/Rpb7 dissociation

Rpb4 and the essential Rpb7 are two subunits that form a heterodimer dissociable from the catalytic core of the RNA polymerase II. RNA polymerases II molecules reside in the nucleus of the cell under all conditions (Farago et al, 2003). These subunits are found in excess with respect to other RNA polymerase II subunits what indicates an additional role for these subunits. It has been proposed that Rpb4 is involved in the communication between the nucleus and the cytoplasmic machineries that determine the levels of mRNAs in the cell (Lotan et al, 2005). Rpb7 also shuttles to the cytoplasm like Rpb4 in response to environmental conditions and it has been described that it is involved in the two mRNA degradation pathways (Selitrennik et al, 2006; Lotan et al, 2007). This heterodimer is thought to couple transcription and translation regulation (Hvel-Sharvit et al, 2010). Rpb7 has been described to physically associate with Tpk2 and Pat1 that are implicated in mRNA degradation (Duan et al, 2013).

Rpb4/7 binds to nascent transcripts (Ujvari et al, 2006) and it is exported from the nucleus to the cytoplasm where it plays a post-trascriptional role (Selitrennik et al, 2006). It also has a role in mRNA degradation through its association with mRNA decay factors and P-bodies (Lotan et al, 2005; Lotan et al, 2007) where those mRNAs that do not engage in translation are accumulated. It has been proposed that this heterodimer associates with mRNA throughout its life cycle, acting as an mRNA coordinator (Halel-Sharit et al, 2010).

1.1.5 Transcriptional stress caused by transcriptional drugs

The effect that these drugs cause is based on the depletion of the level of nucleotide triphosphate (NTPs) in the cell. The NTPs of new synthesis are the essential substrate of the polymerisation reactions carried out by the RNA polymerases. Their levels are carefully regulated by cell growth rates (Uptain et al, 1997). The target of these drugs is the enzyme inositol monophosphate dehydrogenase (IMPDH). This enzyme catalyses a limiting step in the *de novo* synthesis of guanine nucleotides (GTPs). This step consists on the conversion of inositol 5-monophosphate (IMP) to xantosine monophosphate (XMP) with reduction of NAD+. It's an important enzyme which abundance and activity correlates to cell growth rates (Jackson et al, 1975). Its enzymatic and pharmacological characteristics are well-known because it is an excellent target of antiviral and antimicrobial agents, and for antitumoral and immunosuppresor therapies as well (Hedstrom, 1999). It is present in all living organisms. The drugs that can inhibit the function of this enzyme, like 6-azauracil (6AU) and Mycophenolic acid (MPA) (Allison, 2000; Allison and Eugui, 2000) diminish the intracellular levels of GTP and UTP. In yeast, the IMDH coded by *IMD2* is the only resistant enzyme to these drugs. *IMD2* is a gene regulated by a mechanism associated to the election of the transcription start site, what gives as a result a short unstable transcript or a long stable transcript that is translated when the levels of guanine are low (Jenks et al, 2008). Its expression is very dependent on the efficiency of transcription elongation and many elongation factor mutants present a defective activation. As we described above, the mutants of TFIIS are very sensitive to these drugs (Shaw and Reines, 2000). In cells lacking this factor the induction of *IMD2* during treatments with 6AU or MPA does not take place generating a hypersensitivity to these drugs. However, not only the capacity of inducing *IMD2* makes these mutants sensitive to MPA. Its overexpression cannot counteract the effects in some elongation mutants, what suggest that these drugs affect these mutants in a more general way during these processes (Shaw et al, 2001). The addition of 6AU or MPA can diminish RNA polymerase II transcription rates *in vivo,* an effect that only mutants in the polymerase itself present because in general mutants affected in

elongation factors limit exclusively elongating RNA polymerase II processivity and not its transcription rate (Mason and Struhl, 2005).

1.2 The eukaryotic ribosome and its biogenesis

Ribosomes are complex, essential and universal ribonucleoprotein particles that catalyse the polymerisation of amino acids into proteins. This polymerisation is carried out by the decoding of the genetic information contained in the sequence of nucleotides of the mRNA, in a process known as translation (Green and Noller, 1997). The ribosomes constitute the main core of the translational machinery in all the organisms, participating in two fundamental functions: the decoding of the genetic message and the synthesis of proteins. In eukaryotes ribosomes possess a sedimentary coefficient of 80S and it is formed by two ribosomal subunits, 40S and 60S that assemble on the mRNA to initiate translation (Green and Noller, 1997). The small subunit 40S is formed in *S. cerevisiae* by ribosomal RNA 18S and 32 ribosomal proteins (RPs). The 40S subunit has the decoding function and it binds autonomously to the mRNA. It is responsible of keeping the translational fidelity by securing the correct pairing between the mRNA and the tRNA in the denominated decoding centre (Verschoor et al, 1998). The big subunit 60S that in *S. cerevisiae* is formed by the rRNAs 25S, 5.8S and 5S and 46 ribosomal proteins catalyses the formation of peptide bonds between the aminoacyl tRNA and the peptidyl tRNA during protein synthesis elongation, a process that takes place in the peptidyl-transferase centre.

1.2.1 Components of the ribosome
1.2.1.1 Ribosomal RNAs.

In *S. cerevisiae* the ribosomal RNA r(RNA) constitutes the 80% of the total RNA in the cell (Warner, 1999). The small subunit 40S consists of a molecule of rRNA denominated 18S with 1798 nucleotides of length. On the other hand, the big subunit 60S is formed by three molecules of rRNA: 25S that is 3392 nucleotides long, 5.8S of 158 nucleotides and 5S of 121 nucleotides (Mears et al, 2002). Each ribosome possesses equimolar quantities of the four rRNAs.

1.2.1.1.1 Ribosomal DNA transcription

In *S. cerevisiae*, the rRNAs are coded in a unit located in the chromosome XII in a high number of copies. The RNAs 18S, 5.8S and 5S are transcribed by the RNA polymerase I as a 35S precursor of 6.6 Kb that is processed to produce the mature rRNAs. The 5S rRNA is transcribed independently by the RNA polymerase III. It has an extension in the 3' end of approximately 10

nucleotides that is missing in the mature rRNA (Nomura, 2001). In the precursor subunit 35S the sequences that code for the mature rRNAs are separated by two internal transcribed sequences that are called ITS1 and ITS2 and flanked by two external transcribed spacer sequences denominated ETS1 and ETS2. In the 5' and 30 ends of the 5S rRNA are located two non-transcribed spacers NTS1 and NTS2 (Nomura, 2004).

Although the transcription by the RNA polymerase I in *S. cerevisiae* possesses some particularities that are not present in mammals, many of the essential elements of the transcriptional process are highly conserved. The promoter of the 35S transcript consists of 150 base pairs of DNA. In mammals its sequence presents two important elements, the UCE sequence (Upstream Control Element) and the sequence CE (Core Element) (Nomura, 2001). The complex that associate to these elements are denominated UBF (Upstream Binding Factor) and SL1 (Selectivity Factor 1). UBF possesses various HMG1 type DNA binding domains. It is likely that UBF forms a heterodimer that binds both UCE and CE in the promoter region of the rRNA and then recruits SL1 (Moss, 2004). The SL1 complex is formed by 4 proteins: the protein TBP, that binds the TATA box in the same way it does during RNA polymerase II transcription, and three TAFs specific of the RNA polymerase I. After the binding of the UCE and CE complexes, these complexes promote the recruitment of RNA polymerase I via TIF-1A, a homologue of the *S. cerevisiae* protein Rrn3. The phosphorylation of TIF-1A seems to induce the initiation of transcription. Its dephosphorylation during transcription elongation provokes its inactivation and dissociation from the RNA polymerase I (Moss, 2004; Moss et al, 2007; Moss and Stefanovsky, 2002). In *S. cerevisiae* the promoters of the RNA polymerase I also contain the UCE and CE elements although the number of described associated factors is more numerous. For transcription initiation, the RNA polymerase I also interacts with two complexes that are analogue to those of the mammals. The complex UAF (Upstream Activator Factor) is formed by the proteins Rrn5, Rrn9, Rrn10, Uaf30, and the histones H3 and H4, while the complex CF (Core Factor), analogue to the human SL1 (Lalo et al, 1996) consists of the proteins Rrn6, Rrn7 and Rrn11 (Moss, 2004). The UAF complex seems to be involved in the determination of the number of active copies of rDNA (Sanij et al, 2008). This preinitiation complex is completed by TBP and Rrn3 (Nomura, 2001). In *S. cerevisiae* the assembly of the preinitiation complex starts with the recruitment of the UAF complex to the sequence element UE, and it induces the incorporation of the TBP. The binding of the CF complex to the sequence element CE is cyclic during the successive rounds of transcription initiation. The actual model for 35S transcription suggests that UAF recruits CF and finally they bind to the RNA polymerase I. The phosphorylation of Rrn3 regulates RNA polymerase I transcription, (Fath et al, 2001;

Milkereit and Tschochner, 1998). The CF complex is released from the promoter after each round of transcription like Rrn3 in the same manner than in mammalian cells (Moss et al, 2007). The release of Rrn3 does not affect the elongation but it allows the negative regulation of the process. There is no consensus about the possible existence of a regulated promoter escape step during RNA polymerase I transcription initiation (Dvir, 2002; Stefanovsky and Moss, 2006). There are factors like Hmo1 (UBF in mammals) that bind to the elongating form of RNA polymerase I stimulating transcription although their function is not well-known yet (Gadal et al, 2002; Panov et al, 2006). In the last years there have been described many RNA polymerase II elongation factors that are involved in RNA polymerase I transcription also. It seems that DSIF could play a role in RNA polymerase I transcription and in the regulation of rRNA biogenesis (Schneider et al, 2006). Apparently, the PAF complex also plays a role in RNA polymerase I transcription, increasing its transcription rate and connecting with regulatory elements of the TOR pathway (Zhang et al, 2009).

Relative to RNA polymerase I termination sites, there are two termination sequences in vertebrates and yeast (Nomura, 2004). Approximately, the 90% of the transcripts end in a region located 93 nucleotides downstream the 3' end of the mature 25S rRNA. Those RNA polymerases that do not recognise this site reach another position located 250 nucleotides downstream the 3' end of the mature 25S rRNA. *In vitro* and *in vivo* experiments indicate that the Reb1 homologue Nsi1, a factor involved in silencing of rDNA binds to an 11 base pair sequence of nucleotides and provokes a pause of the RNA polymerase I that cause the release of the transcript (Reiter et al, 2012; Merkl et al, 2014).

The transcription of the rRNA 5S is carried out by the RNA polymerase III, that also transcribes the genes of tRNAs and some snRNAs involved in reactions of intron maturation in mRNAs (Ramakrishnan and White, 1998). All these genes present the common characteristic of being small (around 400 base pairs). After elongation, this RNA polymerase recognises as a transcription termination signal a sequence rich in thymines (Schramm and Hernández, 2002). The promoters that recruit the RNA polymerase III can be divided in three groups. The groups of promoters 1 and 2 are characterised for the presence of key sequences that regulate the activity and the recognition of the promoter and are inserted inside of the gene, not possessing TATA boxes. The group 3 of promoters present regulatory sequences in the 5' region of the gene and possess a TATA box. The promoter of 5S belongs to group 1. This promoter consists of an A sequence, an intermediate element (IE) and a C sequence. The A sequence shows a high homology with the sequence present in the promoters of tRNAs (Ciliberto et al, 1983) while the IE and C sequence are specific elements of the 5S rRNA

promoter that play a role in the direct recruitment of the transcription initiation factor TFIIIA (Pieler et al, 1987). These elements constitute the internal control region (ICR) (Pieler et al, 1985). In *S. cerevisiae* only the C sequence is necessary for the active transcription of 5S (Challice and Segall, 1989). The TFIIIA factor recognises the ICR. Its zinc-finger domain allows the formation of a TFIIIA-DNA complex that recruits the TFIIIC factor (Lassar et al, 1983). TFIIIC recruits TFIIIB that consists of three proteins one of them being TBP. The binding of TBP to the promoter recruits the RNA polymerase III. It has also been demonstrated that TFIIS plays a role in the recognition of the transcription start sites in type 3 promoters in a way not related to the domain that is involve in the induction of the RNA polymerase endonucleolytic activity (Ghavi-Helm et al, 2008).

The chromatin organisation of the DNA depends on the activation estate of its copies. Active copies that are being transcribed possess a non-stably positioned nucleosome structure (Dammann et al, 1993) although the pass of the polymerase through these regions depend on the FACT complex that seems to play a role in transcription by the three polymerases (Birch et al, 2009).

1.2.1.2 Ribosomal proteins

Ribosomal proteins are small and basic (with the exception of the acid proteins that form the ribosomal stalk P1A, P1B, P2A, P2B) and they have the capacity of interacting with the rRNAs. Many ribosomal proteins consist of a globular domain that is usually located in the exterior of the ribosome and from that domain emerge a series of prolongations that are inserted in the rRNAs helping to maintain their tertiary structure (Ban et al, 2000; Ramakrishnan and White 1998; Spahn et al, 2001). The ribosomal proteins that interact less with the rRNA only present globular domains and most of them are found on the surface of the ribosomes (Klein et al, 2004). These proteins are essential for the folding of the rRNAs, providing the appropriate structure and support to allow protein synthesis. The big subunit of the ribosome consists of 46 ribosomal proteins and the small subunit of 32 (Wilson and Nierhaus, 2005).

1.2.2   Ribosome biogenesis in *S. cerevisiae*

The formation of the ribosomes starts in the nucleolus where the 35S rRNA is processed to form the mature rRNAs and where different ribosomal proteins that have been imported from the cytoplasm are assembled. During this process different ribosomal pre-particles are

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generated. These pre-particles are transported from the nucleolus to the nucleoplasm and from there to the cytoplasm where they exert their function. All this process is very dynamic. There are many factors that are associated and dissociated after they carry out their function. There are many structural rearrangements and protein exchanges that facilitate the transport of these pre-particles or serve to check the correct assembly of the ribosomes. Ribosome biogenesis consists of two fundamental processes that occur concomitantly:

1- The processing of the rRNA: it starts cotranscriptionally when the 35S precursor is transcribed by the RNA polymerase I and the 5S by the RNA polymerase III in the fibrillar centre and the dense fibrillar component of the nucleolus. The primary transcript is covalently modified by enzymes like 2'-O-ribose-methyltransferases and pseudouridyl syntases, and it undergoes a series of endo and exonucleolytic cleavages in specific sites to produce the mature rRNAs.

2- Ribosome assembly: (for reviews see (Fatica and Tollervey, 2002; Fromont-Racine et al, 2003; Kressler et al, 1999; Venema and Tollervey, 1999). There have been identified approximately 100 snoRNAs and more than 150 trans-acting factors that play a role in ribosome synthesis and cannot be found in the mature subunits. They can be classified in:

   - Ribonucleases. They make cleavages in specific sites of the rRNAs. There are endo and exonucleases 5'-3' and 3'-5'.

   - SnoRNPs. Ribonucleoproteic complexes formed by different factor associated to snoRNAs that are required in different steps of the ribosome biogenesis pathway, including the pseudouridylation of specific residues of the rRNAs and the first steps of their processing.

   - RNA modification enzymes like methyl-transferases, 2'-O-ribose-methyltransferases and pseudouridyl-syntases.

   - RNA helicases. DEAD-box family RNA helicases are the most numerous trans-acting factors that play a role in ribosome biogenesis. It is thought that they intervene in structural rearrangements facilitating the access of the nucleases. They also change the conformation of the RNA, proteins and ribonucleoprotein complexes to allow the association or dissociation of other trans-acting factors or ribosomal proteins and they catalyse the association of rRNAs and snoRNAs (reviewed in (Tanner and Linder, 2001)).

   - RNA chaperones. Facilitate the folding and structural rearrangements of the rRNAs.

- Assembly chaperones or ribosomal protein assembly chaperones. Necessary for the correct association of certain ribosomal proteins.
- Ribosomal like proteins (RLPs). In this category are included some trans-acting factors that possess RNA binding motives very similar to those of some ribosomal proteins, or fragments of sequences identical to the primary sequences of some of these proteins. This suggest that both bind to the same rRNA structures, but RLPs bind to ribosomal pre-particles and ribosomal proteins bind to the mature subunits.
- GTPases and AAA-ATPases. Contribute to the dissociation or incorporation of proteins or also to the rearrangement of the pre-particles before being exported.
- Transport factors. Necessary for the intranuclear transit of the nucleus-cytoplasm transport.
- Many other factors are necessary for the rRNA processing and the assembly of structural elements of the ribosomes but their function is not well known yet (Kressler et al, 1999)

1.2.3 Ribosome biogenesis regulation

The synthesis of proteins is an essential process for all the cells. The cells must control the amount of proteins that are synthesised in response to environmental signals or internal homeostasis. The exact coordination of this process guarantees the success of the cellular division and the survival of the cell. The capacity of protein synthesis of a cell is controlled by the availability of mRNAs, the efficiency of translation and the number of ribosomes (Moss and Stefanovsky, 1995). This number depends on the relative synthesis and degradation rates of proteins, the availability of nutrients in the medium and the growth rate of the cell (Jorgensen et al, 2004). The synthesis of ribosomal subunits is a complex process that require the coordinated synthesis of the ribosomal proteins (RPs), the rRNAs and the processing and assembly of them in the mature ribosomes, a process that is carried out by many factors which expression is also regulated (Grummt, 1999). The synthesis of ribosomes is one of the processes of the cellular metabolism that require a higher energetic use, estimated in an 80% (Hall et al, 2006). This justifies the high regulation of this process. It was calculated that in an actively growing yeast cell, rRNA transcription by the RNA polymerase I represents approximately the 60% of the total transcription in the cell. Additionally, the 50% of the initiation events of the RNA polymerase II occur to generate ribosomal protein mRNAs. 95% of the transcription in the cells is dedicated to these processes. The 90% of the processes of

intron elimination in *S. cerevisiae* occur in ribosomal proteins mRNAs. 25% of the transcription in the cell is dedicated to the production of proteins directly related to ribosomes and translation (Hall et al, 2006; Warner, 1999). Ribosomal proteins and rRNA must be synthesised stoichiometrically and for this reason ribosome biogenesis is a very tightly regulated process, being fundamental the transcriptional regulation of each of its components.

1.2.3.1 Regulation of rDNA expression

In *S. cerevisiae* the four ribosomal RNAs are coded in a unit of approximately 9.1 Kb that is repeated between 100 and 200 times in the chromosome XII (Petes and Botstein, 1977). Not all the copies of rDNA are actively transcribed. In exponentially growing yeast cells only the 50% of ribosomal genes are transcriptionally active (Nomura, 1999). This percentage descends when the cellular necessities vary. For instance there is a decreased production of rRNA in the stationary phase. In higher eukaryotes this silencing depends on the energetic disposition of the cell and it is mediated by the formation of heterochromatin by the complexes NoRC (nucleolar remodelling complex) and eNoSC (energy-dependent nucleolar silencing complex) (Murayama et al, 2008; Nomura, 2004; Santoro et al, 2002). In superior eukaryotes the transcriptional events for copy are usually stable, so it is the number of active copies what possesses the regulatory capacity. However in yeast although the density of polymerases does not vary much, a decrease in the initiation events accompany the decrease of active copies (Claypool et al, 2004; Peyroche et al, 2000). It has been described that the variation in transcription rates is dependent on HMG proteins, like UBF in humans (Stefanovsky et al, 2001). In *S. cerevisiae,* rRNA transcription rates are directly regulated by different kinds of factors. One of them is the Hmo1 protein that presents certain similarity to the human UBF protein and other HMGB proteins (Albert et al, 2013). Hmo1 associates to rDNA loci along all its extension and it is required for rRNA transcription and processing (Hall et al, 2006) being a key element in the regulation of transcription rates dependent of cellular necessities (Merz et al, 2008). The loss of function of Hmo1 leads to a decrease on the products of RNA polymerase I. Other elements that play a role during the elongation step of RNA polymerase I transcription also affect these rates (Zhang et al, 2009).

1.2.3.2 Ribosomal protein expression regulation, the RP regulon

The 78 ribosomal proteins are coded by 138 genes (101 possess an intron). This means that most of the genes that code for ribosomal proteins are duplicated in the genome. The transcription of the 138 RP genes defines the most evident cluster in the eukaryotic transcriptome (DeRisi et al, 1997) and it is known as the RP regulon. Most of these genes

present in their promoters two binding motifs for the proteins Rap1 and Abf1, although in a lower number for the last one (Lascaris et al, 1999; Rotenberg and Woolford, 1986; Schwindinger and Warner, 1987; Woudt et al, 1986; Yarragudi et al, 2004). These binding motifs are located upstream a thymine rich region that is characteristic of the promoters of these proteins, that result free of nucleosomes thanks to the action of these transcriptional regulators (Lascaris et al, 2000; Knight et al, 2014). Rap1 and Abf1 are both global regulatory factors very abundant in the cell that possess functions related to subtelomeric transcriptional silencing, and the structure and recombination of the telomeres (Moretti et al, 1994; Shore, 1994; Yarragudi et al, 2004).

Rap1 has been the most studied in relation to RP promoters. Rap1 is a DNA binding protein that plays a role in numerous processes. It is responsible for the transcription of different genes, from translation factors to glycolysis enzymes (Tornow et al, 1993). Rap1 possesses independent functional domains that have been associated to its different functions. The DNA binding domain is located between the N and C terminal regions but the flanking regions also collaborate in this union (Graham et al, 1999). When Rap1 does not bind to an RP gene promoter the transcription of that gene decreases around a 75% because the thymine rich elements are not enough for its correct activation (Zhao et al, 2006). Rap1 is the responsible for the union of TFIID to these promoters (Mencia et al, 2002), but there are other transcription factors specific of RP genes that cooperate with or depend on Rap1 to carry out the regulated expression of the genes of the RP regulon. The list of these factors consists of Hmo1, Fhl1, Ifh1, Crf1 and Sfp1 (Hall et al, 2006; Jorgensen et al, 2004; Lee et al, 2002; Martin et al, 2004; Rudra et al, 2005; Schawalder et al, 2004).

The protein Hmo1 described in the previous section plays also a positive role in RP genes transcription and its binding depends on the presence of Rap1 with which interacts physically (Berger et al, 2007; Hall et al, 2006). Genomic studies reveal that Rap1 is constitutively present in most of the promoters of RP genes together with Fhl1 (Lee et al, 2002; Schawalder et al, 2004; Wade et al, 2004; Zhao et al, 2006). Fhl1 is a non-essential transcriptional factor that binds RP genes promoters, around 50-100 nucleotides away from the Rap1 binding motif. The union of Rap1 to the promoter region of RP genes is necessary for the union of Fhl1. It is thought that it causes a torsion in the DNA that is required for this union. The relaxing of the repressor action of the nucleosomes present at the promoter is mediated by the histone acetylase Esa1 (Reid et al, 2000; Zhao et al, 2006). The key activator factor of RP gene transcription is Ifh1 (Schawalder et al, 2004; Wade et al, 2004). The union of Ifh1 to its target promoters depends on its physical interaction with Fhl1 because Ifh1 cannot

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bind DNA (Cherel and Thuriaux, 2001; Rudra et al, 2005; Wade et al, 2004). The physical interaction between these two factors activates the transcription of RP genes (Zhao et al, 2006). The repression of RP genes correlates with a loss of Ifh1 but not Rap1 or Fhl1 from the promoters. The binding of Rap1 to DNA in activating conditions recruits Fhl1, keeping the region nucleosome free. Then Sfp1 and Ifh1 are recruited to Fhl1 conforming the FIS complex that recruits Hmo1 and leads to activation (Knight et al, 2014; Reja et al, 2015). Under repressive conditions (for instance during carbon source starvation), Ifh1 interchanges with the Crf1 protein, that plays a repressive role. This interchange provokes the cease of RP genes transcription in a process mediated by the histone deacetylase Rpd3. It is not clear whether the interchange between Ifh1 and Crf1 is because of the competence with Crf1 when it enters the nucleus as a result of the inhibition of the signalling pathway mediated by TOR (target of rapamycin) (Martin et al, 2004; Rudra et al, 2005; Schawalder et al, 2004; Wade et al, 2004). Double hybrid assays have demonstrated that Fhl1 directly interacts with Ifh1 and Crf1. It seems that these two factor share the same binding site to Fhl1, which consists in an FHA (Forkhead Associated Domain) of protein interaction (Martin et al, 2004; Zhao et al, 2006) and this is due to the fact that they proceed from an ancestral common protein and resulted from the divergence after the duplication of *S. cerevisiae* genome (Wapinski et al, 2010).

The functions of all these factors are ultimately regulated by general signalling pathways like TOR or Ras/PKA by a series of integration and transmission elements. These proteins play an important role in the process of activation of RP genes promoters, like Sfp1 and Sch9 (Jorgensen et al, 2004). Sfp1 also plays an important role in the control of cell size and it is an essential element in the transcriptional regulation of elements of ribosome biogenesis (Jorgensen et al, 2004). Sfp1 regulates the expression of RP genes in response to nutritional or environmental changes (Marion et al, 2004) as well as the ribosome biogenesis process itself (Fingermann et al 2003). Its delocalisation out of the nucleus responds to numerous stimulus and it directly affects the subcellular localisation of Fhl1 and Ifh1 as well as their union to the promoters that they regulate (Jorgensen et al, 2004; Urban et al, 2007). In wild type cells Ifh1 and Fhl1 are present in the cell nucleus while in a strain where *SFP1* has been completely deleted there is an enrichment of them in the nucleolus (Jorgensen et al, 2004). Sfp1 changes its localisation from the nucleus to the cytoplasm in a way dependent of the protein Mrs6, that phosphorylates it integrating this way the information that corresponds to the secretion and ribosome biogenesis, acting on TOR1C and regulating the phosphorylation of Sch9 by this complex. However, unlike Sch9 it does not respond to certain stress conditions like osmotic stress or nutrient starvation which suggests additional functions for this factor

(Lempiainen et al, 2009; Singh and Tyers, 2009). Sch9 is a kinase of the AGC family, homologue to Akt/PKB in mammals that possess a C2 lipid binding domain. The kinase activity is essential for its function. It is considered that Sch9 together with Sfp1 play a role in the regulation of RP gene transcription. The loss of function of Sch9 after a treatment with an inhibitor of its kinase activity provokes the immediate repression of the RP and RiBi (ribosome biogenesis) regulons (Jorgensen et al 2004; Urban et al, 2007). Apart from these regulatory pathways there must exist others for instance a direct function of Sch9 on RP genes transcription probably by Rap1 phosphorylation (Jorgensen et al, 2004). The high degree of regulation of these genes gives them unique characteristics, like their low level of biological noise (Newman et al, 2006). On the other hand, the post-translational regulation of RP genes with a high degradation rate dependent on the proteasome completes these proteins finely-tuned regulatory system (Perry, 2007) essential for the perfect coordination between the quantity of ribosomes and the growth of the cell.

1.2.3.3 Transcription regulation of ribosome biogenesis factors: the RiBi regulon.

Genomic approaches have demonstrated that the genes that code for the ribosomal proteins are coordinately regulated with many of the genes that code for factors involved in ribosome biogenesis and both are regulated in response to growth stimuli, or environmental stress among others under the action of Sfp1 which deletion affects RiBi genes even more than RP genes (Gasch et al, 2000; Hughes et al, 2000; Jorgensen et al, 2004; Miyoshi et al, 2003; Wade et al, 2001; Wade et al, 2004). The genes involved in the process of ribosome biogenesis present similar gene expression profiles under different conditions and for this reason these genes have been classified in a group denominated RRB (ribosome and rRNA biogenesis) or RiBi (ribosome biogenesis) regulon that is different to the RP regulon but functions in parallel. The RiBi regulon is formed by at least 200 genes involved in the synthesis of ribosomal subunits such as the genes that code for the RNA polymerase I and III transcription factors, RNA helicases and other RNA modification enzymes (Wade et al, 2006). Most of the promoter sequences of these genes have in common the PAC and RRPE sequence motifs that contribute to their coordinate expression (Wade et al, 2001). There have been identified factors that bind these motifs, like Stb3 that binds RRPE and it is related to the histone deacetylase complex Sin3/Rpd3 (Liko et al, 2007) and the factor Dot6 that binds PAC elements (Badis et al, 2009; Zhu et al, 2009) and also has functions related to nucleosome positioning in these promoter regions.

The regulation of RP and RiBi regulons goes further from the control of the initiation events. In yeast cells growing in rich glucose medium with a functional PKA pathway the levels of active polymerases in RP genes measured by run-on are lower than those expected from the total levels of polymerases detected by ChIP (Pelechano et al, 2009). The ratio of active polymerases versus total polymerases increases when the cells are transferred to a medium with a carbon source like galactose. The same change occurs in RiBi genes but the contrary happens in genes related to mitochondria. This demonstrates that the transcriptional regulation of many genes including RP and RiBi genes also occur by post-initiation events. In the case of RP genes the proportion of total and active polymerases at the 5' end with respect to the 3' regions is higher than the average of yeast genes (Rodriguez-Gil et al, 2010). These polymerases are canonically phosphorylated in serine 5 but their levels of serine 2 phosphorylation are low when the cells grow in glucose. The differences in the phosphorylation and the proportion of active polymerases are mediated by Rap1, in concrete by its SIL domain, implicated in transcriptional silencing in subtelomeric regions (Pelechano et al, 2009). The detailed mechanism of this additional level of transcriptional regulation is not well understood yet.

1.2.3.4 Coordinated regulation of the three polymerases

The RP and RiBi regulatory network has links to RNA polymerases I and III transcription. Many factors that participate in the regulation of different polymerases have been described and proposed as active elements of coordinated regulation of them. An example is Hmo1 that seems to play a relevant role in RP genes promoters apart from regulating rRNA transcription rates (Gadal et al, 2002; Hall et al, 2006). Rpd3 also binds RP genes promoters and rDNA (Humphrey et al, 2004; Rohde and Cárdenas, 2003; Tsang et al, 2003) and Abf1 seems to have rDNA related functions although in this case related to the delays of the ARS (autonomous replicating sequences) of these regions (Muller et al, 2000). It has been observed how the deletion of *FHL1* causes a six-fold decrease in the levels of 25S and 18S mature rRNAs with respect to the levels of tRNAs (Rudra et al, 2005).

Transcription is not the only process that is coordinated. It has been described a complex formed by Ifh1, CK2, Utp22, and Rrp7. Rrp7 and Utp22 play important roles in the processing of the rRNA and Ifh1 is an essential activator of RP genes transcription. Fhl1 but not Rap1 interacts with this complex and it interacts through its Ifh1 binding domain. This complex is denominated CURI and it could recruit positive factors for RP, RiBi and rDNA, coordinating

the global process of ribosome biogenesis with the expression of the genes that code for their structural elements (Rudra et al, 2007).

1.2.3.5 Regulation of ribosome biogenesis under stress conditions

The TOR signalling pathway also regulates RP genes transcription in response to nutrient availability. This pathway is antagonistic to the Ras/PKA pathway. The inhibition of the TOR pathway by rapamycin causes the same changes in the localisation of Crf1 than nutrient starvation. TOR regulates the localisation of Crf1 by its action on the Yak1 kinase via PKA (Martin et al, 2004). Nutrient starvation and the lack of a nitrogen source in the medium activate TOR that regulates RP gene transcription controlling the cellular localisation of PKA and the kinase Yak1, regulated by PKA. In this mechanism take part the transcriptional factor Fhl1 and two cofactors, IFh1 (co-activator) and Crf1 (co-repressor). When the cells detect changes in the availability of carbon or nitrogen in the medium the Ras-cAMP/PKA pathway is activated which results in the coordinated inhibition of RP genes and rRNA transcription.

The intracellular lack of aminoacids leads to the derepression of Gcn4 transcription that acts as an inhibitor of RP genes, probably in an indirect way sequestering one or more of their transcriptional factors (Hinnebusch and Natarajan, 2002; Moehle and Hinnebusch, 1991; Natarajan et al, 1999; Natarajan et al, 2001).

Heat stress by sudden increases in temperature cause in *S. cerevisiae* the repression of the genes that code ribosomal proteins, decreasing the quantity of their mRNAs for approximately 30 minutes and recovering the normal levels after 1 hour (Eisen et al, 1998; Li et al, 1999; Miyoshi et al, 2003; Warner, 1999; Warner and Gorenstein, 1977).

Finally, the secretion pathway in *S. cerevisiae* is essential for the growth of the plasma membrane and the cell wall. The synthesis of proteins in cells with defects in the secretion pathway causes an internal pressure that dilates the plasma membrane, a signal that is detected by Wsc1 (Gray et al, 1997). It activates the protein kinase C (PKC) through a series of intermediates provoking a decrease in the synthesis of ribosomes and tRNAs (Li et al, 2000; Mizuta et al, 1998; Nierras and Warner, 1999). The repression of RP genes mediated by this signalling pathway also depends on the SIL domain of Rap1. Some mutations that cause alterations in the synthesis of 60S subunits inhibit this repression of the transcription of RP genes and rRNAs provoked by a defect in the secretion pathway. However it does not happen in the case of 40S subunits suggesting that the disassembly of defective 60S subunits suppress the signal generated by a defect in this pathway (Deloche et al, 2004, Zhao et al, 2003).

1.3   Nucleolar stress

Nucleolar stress refers to those failures in ribosome biogenesis or function that causes disruptions like cell cycle arrest and apoptosis. Eukaryotic cells tightly regulate cell cycle transitions to ensure viability and the correct transmission of genetic information. A fundamental element of cell cycle regulation consists of arrests at particular steps to guarantee the completion of a previous cell cycle event, to repair cellular damage, or to resolve a challenge situation (Hartwell et al, 1994). Failures in these processes reduce cell survival and, in higher metazoans, lead to cancer and other diseases (Hoeijmakers, 2001; Friedberg, 2003; Thompson and Compton, 2001). Ribosome biogenesis is a highly resource consuming process and therefore involves the tight regulation and balanced synthesis of all its constituents (Warner, 1999; Boulon et al, 2010; Rudra et al, 2007). This complicated pathway requires the coordinated assembly of ribosomal RNAs, synthesized by RNA polymerases I and III (Warner, 1999) and ribosomal proteins, whose mRNAs are transcribed by RNA polymerase II. 6AU and MPA are nucleotide-depleting drugs that interfere with transcription elongation *in vivo* by inhibiting the enzyme inosine monophosphate dehydrogenase (IMP), a rate-limiting enzyme in the *de novo* synthesis of guanine nucleotides. These drugs cause nucleolar stress through the accumulation of free ribosomal proteins. In mammalian cells, the accumulation of some specific free ribosomal proteins results in the activation of p53, leading to cell-cycle arrest and apoptosis (James et al, 2014). Our laboratory has demonstrated that NTP depleting drugs also induce ribosomal protein accumulation and nucleolar stress in yeast cells, which lack p53 and its critical E3-ubiquitin ligase MDM2 (Gómez-Herreros et al, 2013).

2. Nuclear localisation of genes inside eukaryotic cells

2.1 Structure of the yeast cell nucleus

Eukaryotic cells carry their genetic material in the nucleus, an organelle enclosed by a double membrane contiguous to the endoplasmic reticulum that communicates with the cytoplasm through a system of nuclear pores that control the traffic of molecules. The basic principles of nuclear organization can be observed from yeast to human. The study of nuclear organization in yeast allows the use of easy genetics and techniques like live microscopy to understand its structures. However, there are features that are characteristic of certain tissues or species. The yeast nucleus, although being a typical eukaryote possesses some distinctive characteristics. In yeast, the spindle pole body (SPB) is located embedded in the nuclear envelope. The SPB is one of the structures in the nuclear envelope where chromatin can be anchored, together with the nuclear pore complexes (NPCs). The SPB localises opposite to the

nucleolus in yeast, and this position is determined by the emergence of the bud at the moment of budding. The nucleolus is formed by the rDNA locus region of chromosome XII that contains tandem repeats copies of the DNA that codes for ribosomal RNA. The yeast nucleolus has a crescent shape and its size is about one third of the nuclear volume (Léger-Silvestre et al, 1999). The nucleolus originates from rDNA by self-organisation (Misteli 2007; Hernández-Verdún, 2006). The centromeres are another one of the nuclear features that determine chromatin architecture in the yeast nucleus. The positioning of chromosomes inside the yeast nucleus is constrained in the first place by the attachment of centromeres to the SPB and telomeres to the nuclear envelope (Therizols et al, 2010). In yeast, the nucleus maintains a Rabl-like organization, a rosette like structure clustered around the SPB to which the centromeres are attached through microtubules (Rabl, 1885). The binding of centromeres to the SPB is maintained during all the cell cycle. The ends of short chromosome arms cannot explore the nuclear periphery, and are confined to a region opposite the nucleolus. The contact between chromosomes occurs in a window of 20 kilobases around the centromeres (Duan et al, 2010). Another feature of the yeast nucleus organisation consists on the clustering of the 32 yeast telomeres in a number of foci (3-6) at the nuclear envelope (Palladino et al, 2003). These telomeric foci are dynamic, moving in a random motion (Therizols et al, 2010).

2.2 Organization of the yeast nucleus

These three structural elements, the centromeres, the telomeres and the nucleolus play a role in the organization of the nucleus in yeast (Taddei and Gasser, 2012; Berger et al, 2008). Genomes are not organized as sequences, but instead they are elaborate physical structures which organization plays a role in different processes such as transcriptional regulation or DNA replication. Chromosomes in metazoans occupy distinct regions denominated chromosome territories (Branco and Pombo, 2006). In yeast different studies indicate the existence of similar territories (Lorenz et al, 2002; Berger et al, 2008; Liti et al, 2009; Duan et al, 2010). The dynamics of the chromosomes can be approximated by the motion of an entropic spring (Verdaasdonk et al, 2013). It has been hypothesized that the tethering of a chromosome arm could be detached in case of DNA damage. This would allow the movement of the chromatin for its repair. In this way, the detachment of a chromosome end would allow the chromatin to explore a larger region of the nucleus (Verdaasdonk et al, 2013).

2.3 Consequences of nuclear organization

There are different processes affected by the nuclear organization of the chromatin. In the first place, the most evident effect is the silencing in the subtelomeric regions of the chromosomes caused by the enrichment on Sir proteins that provoke repression. This recruitment is dependent on the DNA sequence. Telomeres in yeast consist of TG-repeats that extend for various kilobases silencing the neighbouring promoters. The repression of these regions requires the binding of SIR proteins, the trimeric complex formed by Sir2, Sir3, and Sir4 that recognise unmodified nucleosomes to reduce endonuclease accessibility. The silencing at the telomeric regions occurs in the same way at the *HML* and *HMR* silent mating-type loci (Rusche et al, 2003). Apart from the repression of subtelomeric regions, the recruitment of SIR proteins also prevent the repression of different promoters located at other locations in the genome, for instance, the release of SIR proteins from their foci affects ribosome biogenesis genes what could be part of a regulatory mechanism that involves the derepression of genes that are silenced in normal conditions and the transcriptional repression of ribosomal genes with the object of decrease growth rate (Taddei et al, 2009).

On the other hand, NPCs bind active genes. The mechanism that produces the relocation of genes to the NPCs upon activation is yet unknown. This activation seems to be especially important for genes with galactose or heat-shock induced promoters. Being located close to the NPC these genes are assured a quick expression and mRNA export and also their quick reactivation after repression.

2.3.1 Spatial localization and transcription

The spatial localization of a gene inside cell nucleus is implicated in the regulation of transcription. Several studies link subnuclear gene location to gene activity (Misteli et al, 2007). The nuclear pore complex seems to play a role anchoring highly active genes, which is especially important in the case of inducible genes like *GAL1* or heat shock genes (Casolari et al, 2004, 2005; Schmid et al, 2006; Cabal et al, 2006; Burns nd Wente, 2014), a process known as gene gating, providing an environment that favours transcriptional activation in contrast to the zones located between NPCs that would be more repressive. The spatial distribution of the sixteen chromosomes of *Saccharomyces cerevisiae* is not random, although the chromatin experiments continuous movement (Marshall et al. 1997; Gasser 2002). It seems that transcription involves certain degree of movement. This movement is sensitive to glucose concentration in the medium and concentrations of ATP (Heun et al, 2001) although the enzymes responsible are unknown. The need of ATP suggests that this movement is active or non-Brownian. In human cells it has been observed that during strong transcriptional

activation the movement of chromatin is directional and not random. Also in *Drosophilla* it could be observed a non-random movement during spermatocyte differentiation (Vazquez et al. 2001; Chuang et al. 2006). Nucleosomes remodelers dependent on ATP play a role in chromatin that is going to be transcribed, altering nucleosome position and accessibility for DNA-binding factors (Flaus and Owen-Hughes 2004; Clapier and Cairns 2009). It has been shown that the remodeler complex INO80 is related to an increased chromatin mobility (Neumann et al, 2012). The mechanisms of chromosome dynamics are unknown in yeast. In mammalian cells actin and myosin motors play a role in movements that accompany transcriptional activation (Chuang et al, 2006; Dundr et al, 2007). The mechanisms for gene relocation could involve molecular motors, or alternatively, it could be a result of changes in the chromatin organisation, for instance a decondensation of the chromatin fibre. In this case it would depend on the local effect of remodelers on nucleosomes.

2.4 Visualization of the yeast nucleus

The small size of the yeast nucleus (1-1.5 μm) is a limiting factor in the study of nuclear organization. The resolution of conventional light microscopy is not enough to distinguish its features. Many different techniques have been developed over the past few years that have allowed the analysis of nuclear organization. Techniques such as chromosome conformation capture (3C), chromosome conformation capture on chip (4C), chromosome conformation capture carbon copy (5C), ChIA-PET and HiC (reviewed in de Wit and de Laat, 2012) permit the generation of DNA interaction maps. But these techniques don't allow the detection of single cell variation. Recently, the development of new imaging techniques has allowed the mapping of the yeast nucleus at better detail, although the image studies can suffer from a series of limitations. In the first place, the limited resolution of optical microscopy limits the observation of nuclear subcompartments due to the small size of yeast nuclei. Also, these techniques only allow the observation of a low number of cells. The study of nuclear compartmentation must account for cell to cell variability. This makes necessary to analyse a high number of cells in order to obtain statistically significant data. Finally, many studies measure locus position only in terms of relative distance from a nuclear landmark such as the nuclear centre but fail to acknowledge the geometry of the cell nucleus. There have been developed new techniques that with the help of new imaging technologies allow the generation of high-resolution probabilistic maps considering thousands of cells. The use of this technique has proved further the existence of chromosome territories in yeast (Berger et al, 2008).

2. OBJECTIVES

This thesis is based on the previous work performed by Fernando Gómez-Herreros on the effect of transcriptional stress on the genes involved in ribosome biogenesis in *Saccharomyces cerevisiae.* During that work, the involvement of TFIIS and RNA pol II backtracking in that phenomenon was described. In this thesis we continue that study, addressing the following aims:

1- To understand the role of TFIIS in response to nucleolar stress.

2- To investigate the occurrence of RNA polymerase II backtracking across the genome and its function in gene expression.

3. RESULTS

Chapter 1

The role of TFIIS in nucleolar stress

3.1.1   TFIIS effect on RNA polymerase activity.

RNA polymerase II arrest and backtracking are very frequent phenomena (Galburt et al, 2007; Churchman and Weissman, 2011), what suggests that spontaneous non-stimulated RNA cleavage is sufficient for sustaining gene transcription under standard yeast growing conditions (Sigurdsson et al, 2010). However, the yeast *dst1Δ* mutants lacking TFIIS are highly sensitive to drugs that impair the *de novo* synthesis of nucleotide triphosphates (NTPs) such as 6-azauracile (6AU) and mycophenolic acid (MPA) (Exinger and Lacroute, 1992). In response to 6AU or MPA, yeast cells up-regulate the expression of *IMD2*, a gene encoding an IMP dehydrogenase isoenzyme that is resistant to such drugs (Shaw and Reines, 2000). The mutant *dst1Δ* is unable to up-regulate *IMD2* (Shaw and Reines 2000), which exhibits a sophisticated transcriptional attenuation mechanism in response to GTP levels (Kuehner and Brow, 2008). The over expression of *IMD2* suppresses the MPA sensitivity of yeast MPA-sensitive mutants (Desmoucelles,et al, 2002). The transcriptional stress caused by NTP-depleting drugs is partially transient in wild type yeast cells due to the up-regulation of *IMD2*, whereas it is more intense and permanent in *dst1Δ* (Shaw and Reines, 2000). Most of the studies conducted on the TFIIS function have focused on its role during RNA polymerase II dependent transcription. However, it has been shown that TFIIS and TFIIS-like cleavage factors are also important for RNA polymerase I and RNA polymerase III-dependent transcription (Schnapp et al, 1996; Ghavi-Helm et al, 2008). All this information suggests that TFIIS generally contributes to the biogenesis of ribosomes, whose structural elements are concertedly transcribed by the three nuclear RNA polymerases (Warner et al, 2001). In this chapter we demonstrate that TFIIS is required to maintain the transcriptional activity of RNA polymerase II when transcribing ribosomal protein genes (RP genes) under transcriptional stress conditions.

3.1.1.1 TFIIS effect on rDNA

In order to evaluate the contribution of TFIIS to transcriptional activity under transcriptional stress, we monitored its occupancy by performing Chromatin Immunoprecipitation (ChIP) experiments on cultures that were treated with 6-azauracil (6AU). TFIIS was originally described as an RNA polymerase II specific factor, although TFIIS and TFIIS-like functions have been reported to also impact transcription by RNA pol I and III (Schnapp et al 1996; Tschochner et al 1996; Labhart, P 1997; Chèdin et al, 1998). Moreover, it has been demonstrated that TFIIS is bound to any transcribed locus of the nuclear genome, including rDNA regions transcribed by RNA polymerase I (Ghavi-Helm et al, 2008). As a first approach to understanding TFIIS role during transcription we confirmed the binding of TFIIS to rDNA and

noted a significant decrease in the binding of this factor upon 6AU 100 µg/ml treatment (figure 1A). The occupancy of rDNA by RNA polymerase I was determined by the immunoprecipitation of Rpa190, the largest subunit of the RNA polymerase I. The occupancy of Rpa190-HA was transiently influenced by 6AU as can be expected for a stressful situation. However, this response was almost identical in a *dst1Δ* mutant (figure 1B). This result suggests that TFIIS does not play a relevant role in rDNA transcription during NTP depletion.

We also monitored the amount of transcriptionally active RNA polymerases I in a wild type and a *dst1Δ* mutant by transcriptional run-on. The run-on did not show lower levels of active RNA polymerase I in the *dst1Δ* background (figure 1C). The run-on/Rpa190 ratios in a *dst1Δ* background confirm that TFIIS does not play a role in preserving the activity of elongating RNA polymerase I under NTP depletion (figure 1 D).
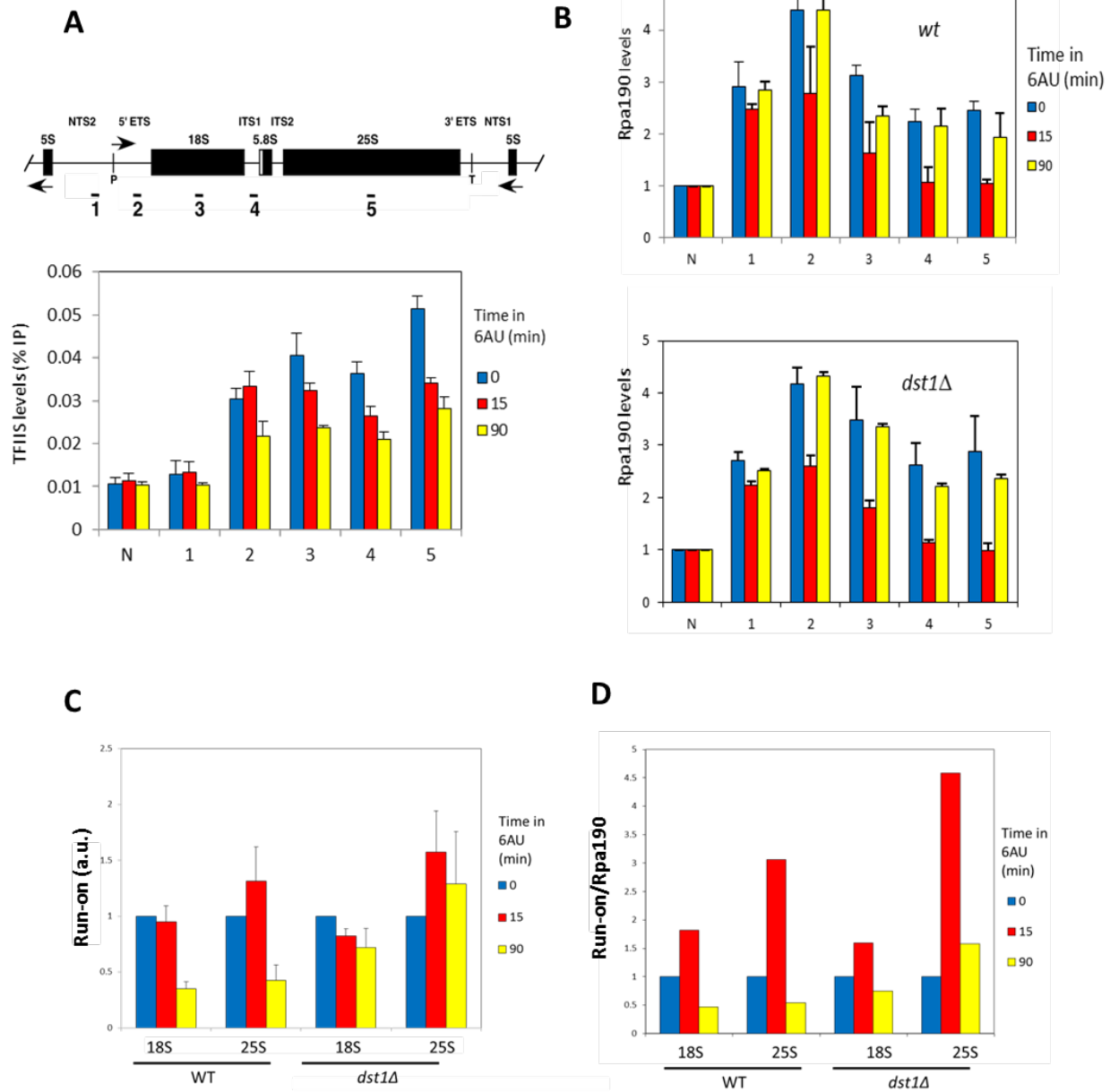
Figure 1: TFIIS in rDNA during NTP depletion. **A** The ChIP experiment was performed using antibodies against an HA-tagged version of TFIIS. The ChIP shows a constant binding of TFIIS that decreases upon 6AU addition. Location of the amplicons used for quantitative PCR is shown above the graph. ChIP signals were quantified in relation to the input material. All the values represent the average of at least three independent experiments. The error bars indicate standard deviation. **B** The variation in the distribution of RNA polymerase I was measured by ChIP experiment using antibodies against a HA tagged version of Rpa190. The data is normalized to a non-transcribed amplicon (N) The transient decrease in the levels of Rpa190 after 6AU addition is independent of the presence of TFIIS. **C** The run-on does not show lower levels of active polymerases in the *dst1Δ* background with respect to the wild type. **D** The ratio Run-on/Rpa190 is not affected by TFIIS.

3.1.2   TFIIS role in ribosomal protein genes expression.

As we did for rDNA, we also detected the presence of HA-TFIIS in the genes encoding ribosomal proteins (RPs) and other highly expressed RNA polymerase II-dependent genes. We measured TFIIS binding at three different positions within the transcribed region of ten genes, four RP genes (*RPS3, RPS8, RPL5* and *RPL25),* two ribosomal biogenesis (RiBi) related genes (*RPA43* and *RRP12)*, and four genes with no direct relation to ribosomes (*ADH1, PHO88, HXT1* and *HXT2).* The TFIIS ChIP signal was consistent and showed intensities that were proportional to the amount of polymerases present in the genes as measured by Rpb3 ChIP (compare figures 2A and 2B). The addition of 6AU 100µg/ml to the cultures led to a decrease of the levels of TFIIS on all the four RP genes, *HXT1, HXK2* and a minor effect on the other four genes (figure 2A). It also caused a rapid decrease of RNA polymerase II occupancy on the RP genes and RiBi across their entire length (fig 2B). However this was not the case for the rest of the genes tested, which showed a milder decrease along the transcribed region, particularly at the 5' end. This effect caused a relative accumulation of polymerases at the 5'-end as previously described (Mason and Struhl, 2005) with no significant decrease along the transcribed region. The comparison of TFIIS and the Rpb3 ChIP results shows a difference between ribosome-related genes and the rest. Whereas RP genes showed parallel changes of TFIIS and Rpb3 signals in response to 6AU, the other genes presented an imbalance between them (figure 2A and 2B).

In order to quantify the impact of 6AU on the transcriptional availability of TFIIS, we calculated the ratio for each gene normalized to the initial value before 6AU addition. The two RiBi genes were omitted because of their low Rpb3 values, which were very close to that of a non-transcribed control (figure 2B). We found that the TFIIS/Rpb3 ratios remain unchanged in the four RPs upon 6AU addition, while the polymerases transcribing the other four non-RP genes became TFIIS-impoverished. This difference was observed along the length of the genes (figure 2C).
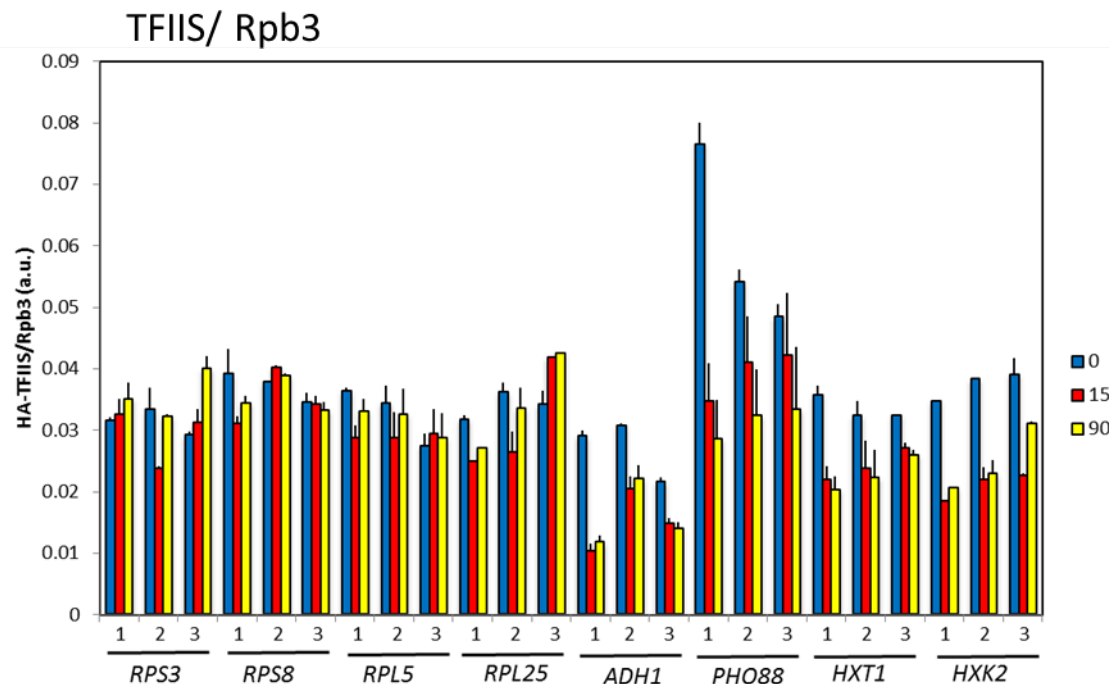
A

TFIIS

B
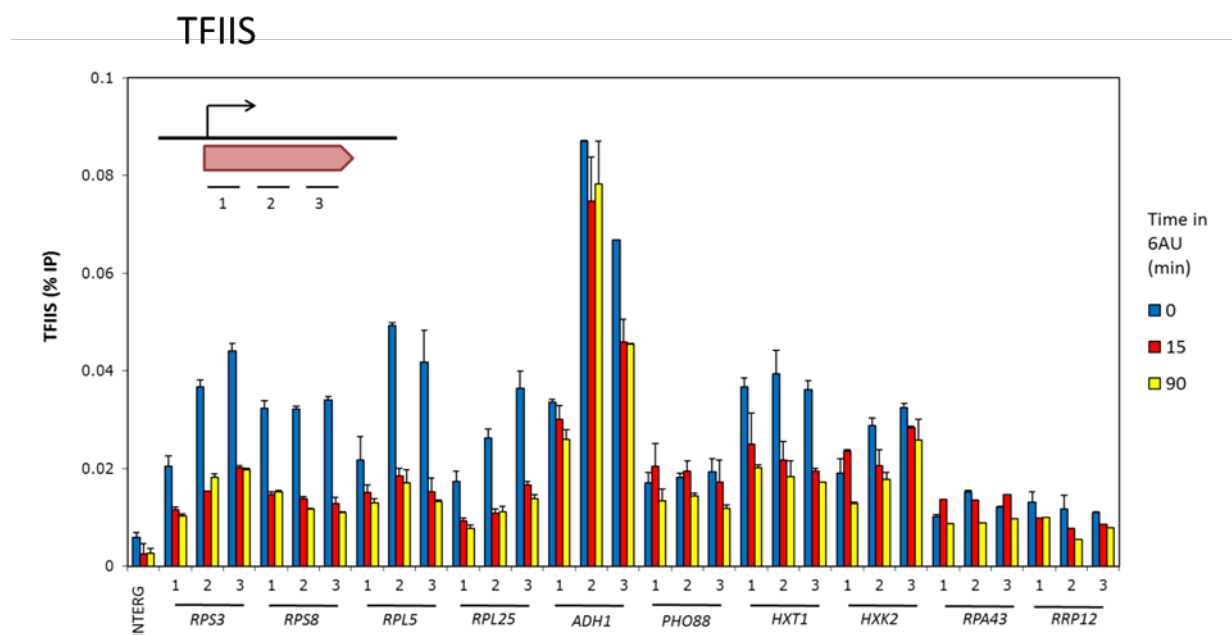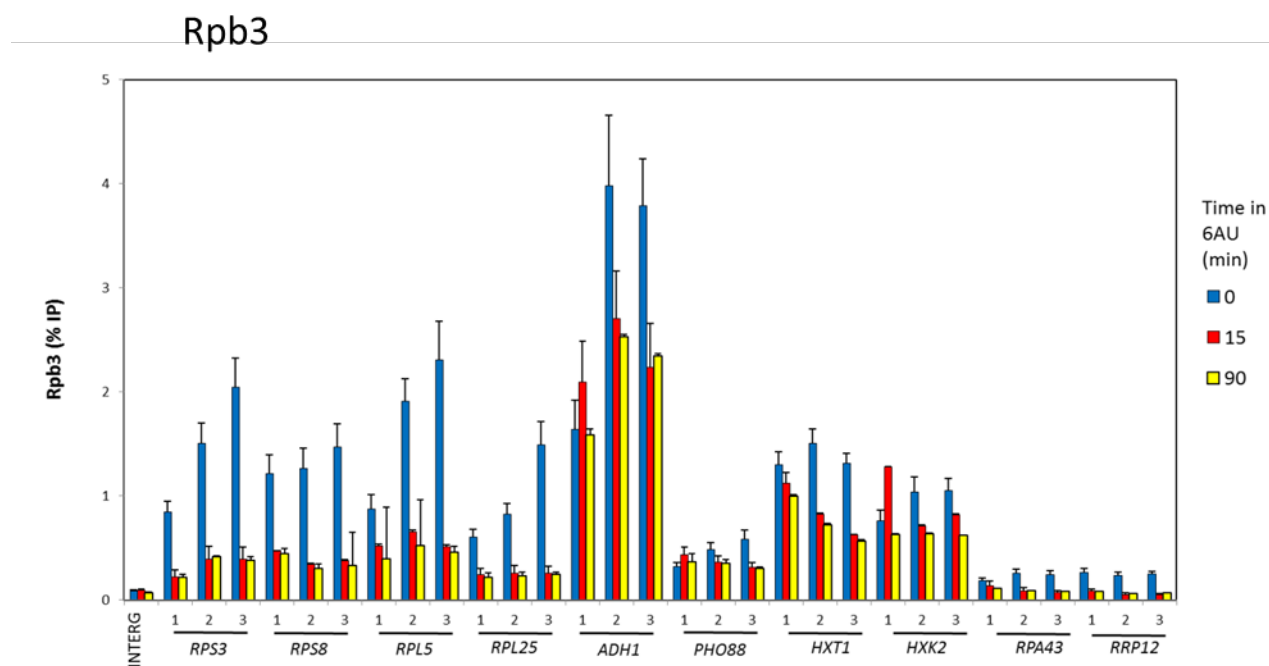
Rpb3

C

Figure 2: TFIIS and RNA polymerase II occupancy in response to 6AU. All the samples were extracted in parallel from the same cultures for the analysis of HA-TFIIS and Rpb3 by ChIP experiments utilising antibodies against HA-TFIIS and Rpb3. ChIP signals were quantified in relation to the input material. The results of an unstranscribed intergenic region (Chromosome V, co-ordinates 9716-9863) are also shown. Error bars indicate standard error. Changes in TFIIS **A** and Rpb3 **B** binding to RNA polymerase II-dependent genes in response to 6AU 100µg/ml. The values represent the average of three independent experiments at three different amplicons distributed along the genes. **C** TFIIS/RNA polymerase II ratios upon 6AU (100 µg/ml) addition at the same amplicons as in **A** and **B**.

3.1.3   RNA polymerases in *dst1*

These results suggest that upon 6AU treatment TFIIS preferentially binds to those polymerases that are transcribing RP genes. In order to investigate whether this phenomenon had any functional influence on RP transcription we studied the variation of RNA polymerase II occupancy in response to 6AU in a *dst1Δ* mutant. We found that RP genes had a slower decrease in RNA polymerase II occupancy in the mutant than in the wild type strain, while non-RP genes behaved similarly in both strains. The difference between the wild type and *dst1Δ* for RP genes was particularly clear at the 5'-end (figure 3A).

We also measured by transcriptional run-on the amount of transcriptionally active polymerases on the different genes tested. This technique allows the detection of those RNA

polymerases actively engaged in transcription, specifically those that do not display a backtracked configuration. As we assume that each active polymerase produces a similar signal in the run-on assay irrespectively of its position in the genome any variation in the run-on signal of a gene would reflect a change in the number of active polymerases that are transcribing such a gene. In the wild type, most of the genes analysed were able to maintain their run-on signal unchanged after 15 minutes in 6AU (figure 4C). In *dst1Δ* all the genes showed decreased run-on signals upon 6AU addition but this decrease was especially intense in the four RP genes (figure 4B).

A
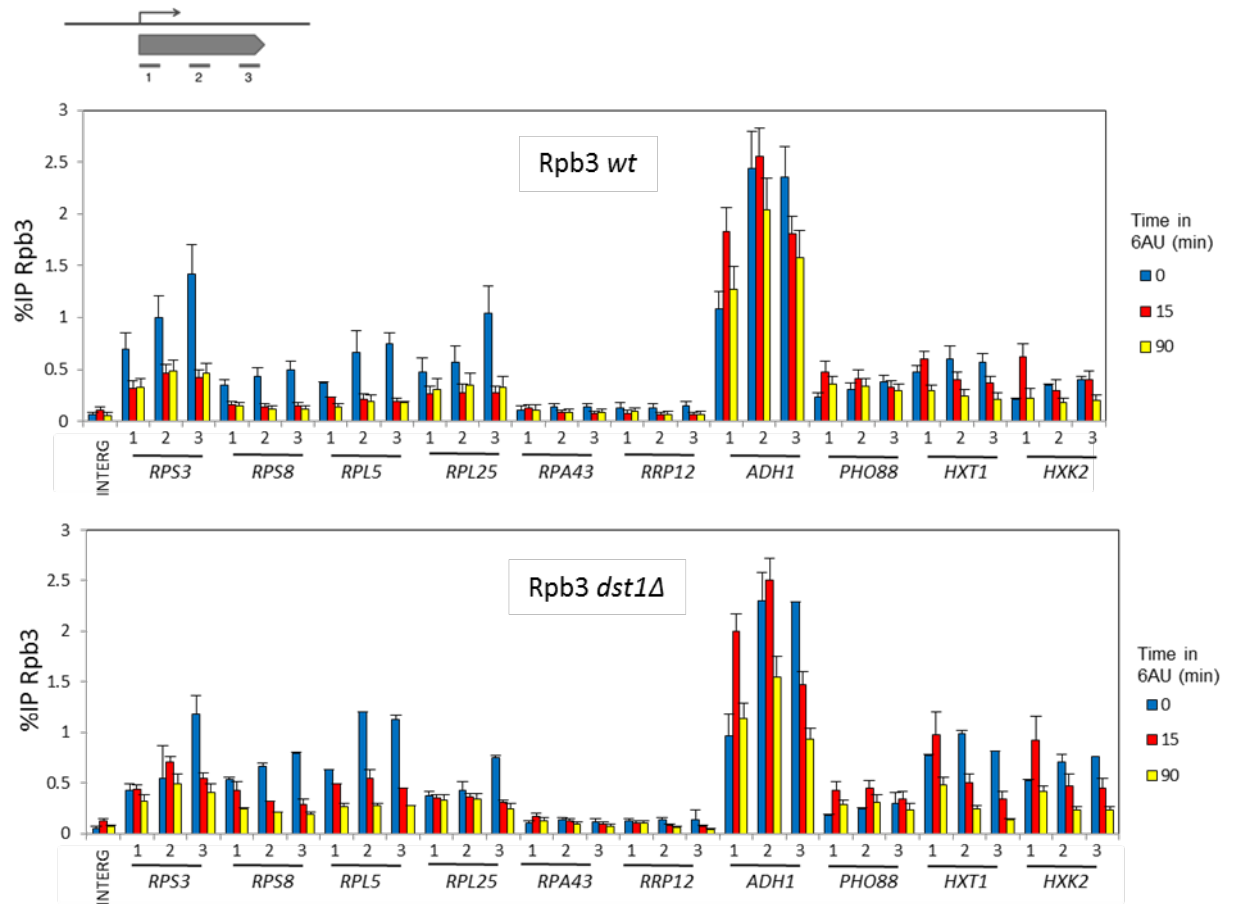
Figure 3 TFIIS sustains RNA polymerase II activity in RP genes under transcriptional stress. Variations in the levels of RNA polymerase II bound to the indicated genes caused by the addition of 6AU to both the wild type and an isogenic *dst1Δ* strain. All the values represent the average of three independent experiments.

The comparison between the Rpb3 ChIP and the run-on results offers additional clues to interpret these experiments. The reduction in RNA polymerase II occupancy exhibited in the wild type by RP genes after 15 minutes in the presence of 6AU (figure 4B) was not reflected in the density of active polymerases measured by transcriptional run-on (figure 4A). The same comparison made in *dst1Δ* offers the opposite outcome for RP genes: a slower decrease of Rpb3 than the run-on signal (figure 4A). These results suggest that TFIIS plays an important role in RP genes during NTP depletion by maintaining their RNA polymerase population fully active. The run-on/Rpb3 ratios calculated for each gene by dividing the signal value obtained from the run-on assay between the value that corresponds to the total level of Rpb3 as measured by ChIP, confirmed a marked increase of RNA polymerase II specific-activity (active transcription/total RNA polymerase II) in the four RP genes upon 6AU addition, which did not occur in non-RP genes (figure 4C). RNA polymerase II specific-activity sharply dropped in the *dst1Δ* cells in all the genes tested upon 6AU addition, confirming that the higher run-on/Rpb3 ratios exhibited by the four RP genes in the wild type depends on TFIIS. We conclude that the sustained TFIIS/RNA polymerase II ratio exhibited by RP genes after NTP depletion is responsible for the high RNA polymerase II specific-activity detected in these genes.
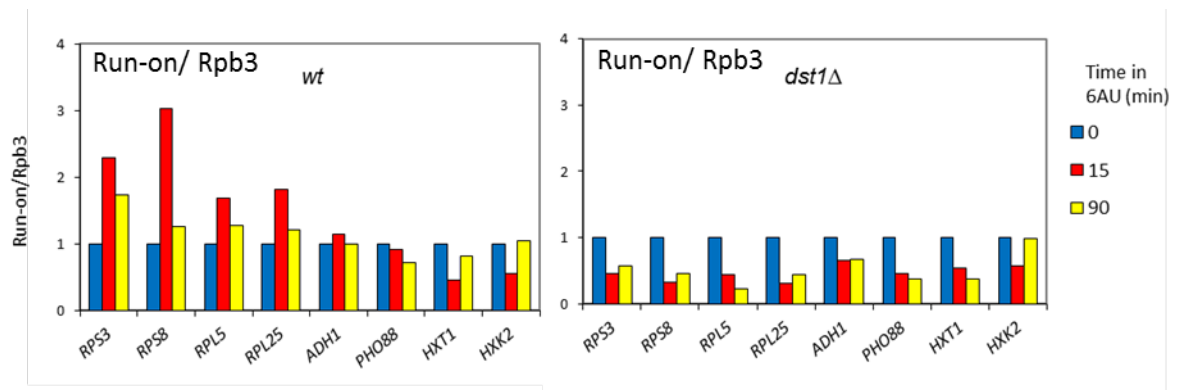
A
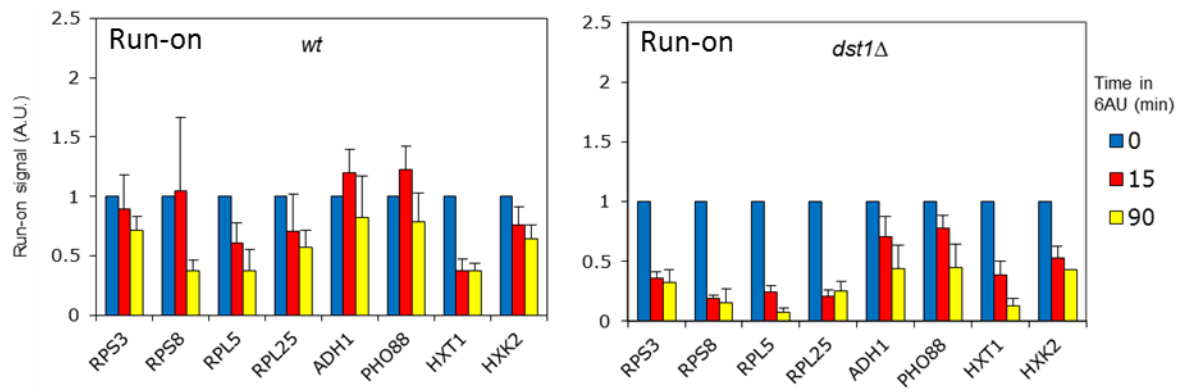
B
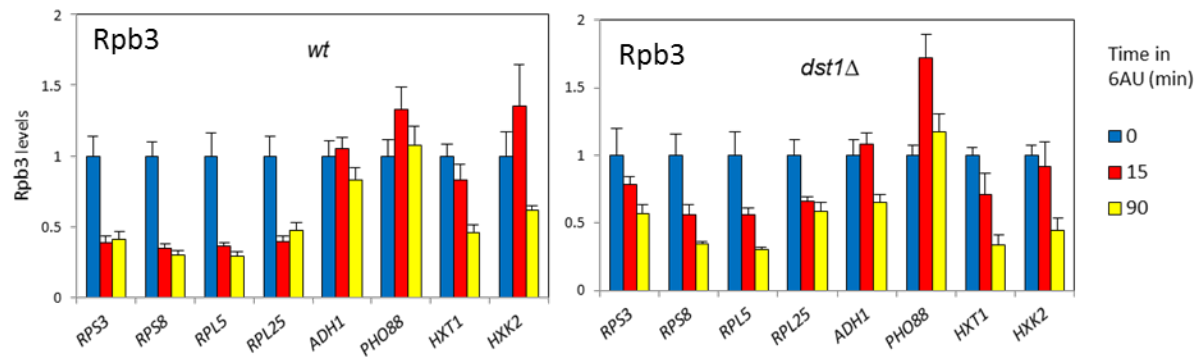
C

Figure 4 A Averaged levels of RNA polymerase II at each gene and (**B**) variations in the transcriptional run-on signal of polymerases sitting on the indicated genes caused by the addition of 6AU 100 µg/ml normalised to time 0 in a wild type and a *dst1Δ* strains. **A** Variations in the specific activity of polymerases on the indicated genes caused by the addition of 6AU 100 µg/ml normalised to time 0 in a wild type and a *dst1Δ* strains. RNA polymerase II specific activity was expressed as the ratio between the variation in transcriptional run-on signal and the variation in Rpb3 ChIP signal shown in (**A**) and (**B**).

3.1.4 RP genes localization during nuclear stress

Results from the previous section show that RNA polymerases are prone to backtrack on RP genes, which makes these genes particularly sensitive to the lack of TFIIS in conditions of transcriptional stress (NTP depletion) being required to maintain RNA polymerase II specific-activity on those genes upon 6AU addition.

It has been described that the spatial localisation of a gene inside the cell nucleus plays a role in the regulation of its transcription, existing studies that link gene localisation to gene expression (Misteli et al, 2007). An association between RP genes (*RPP1A, RPL13A, RPL2A,*

RPL29) and a nuclear pore protein has been established by (Yoshida et al, 2010). Taking this into account were interested in determining if the especial transcriptional behaviour that we observed in RP genes could be related to their localisation within the nucleus. We decided to study whether there is a dependency on TFIIS for the localisation of the RP genes that might be influenced by the transcriptional stress caused by the addition of 6AU.

3.1.4.1   Effect of 6AU in the nuclear localization of *RPS3* and *RPL25* in a wild type background

We decided to study the localisation of the RP genes *RPS3* and *RPL25* within the nucleolus. We made use of the imaging technique that allows the determination of the positioning of different genes inside the nucleus with very high spatial resolution, which was developed by Dr. Olivier Gadal and collaborators (Berger et al, 2008). Using Fluorescent Repressor Operator System (FROS), TetO repeats are inserted near any gene of interest in a yeast cell expressing the nuclear pore protein Nup49 fused to GFP to allow the visualisation of the nuclear envelope and the nucleolar protein Nop1 fused to mCherry labeling the nucleolus. To analyse the spatial location of a gene locus in the nucleus of *Saccharomyces cerevisiae* the three dimensional position of said locus relative to the nuclear envelope, the nuclear centre and the nucleolus is computed from a large number of individual nuclei (typically over 1000 nuclei). For this experiment asynchronous live-cell populations are imaged in three dimensions (3D) using confocal microscopy, each image consisting of approximately 200 cells. Then an automated module identifies those cells in interphase (G1 and S phase) in the fluorescence image. For each individual cell are automatically computed the 3D coordinates of the locus, the nuclear centre and the nuclear "centroid" and high-resolution probabilistic gene maps are generated from these computed distances. This distribution is described as gene-map. The probability density of gene position is plotted relative to median (in the analysed cell population) nuclear envelope and nucleolus. To obtain the labelled strains, we followed the protocol established by (Berger et al, 2008) (see Materials and Methods). All the constructions were validated by PCR and the intensities of the fluorescence signals were checked by microscopy before carrying out the analyses. In order to obtain consistent results, at least three independent experimental replicates were performed for each strain. A summary of the results is shown in figure 5A. The y-axis represents the cumulative distribution of the total population of cells analysed while the x-axis shows the distance between the gene and the centre of the nucleolus measured in μm. In a wild type strain the localisation of *RPS3* appears

to vary during a treatment with 6AU. If we compare the distance at time 0 (black lines) with the curves generated after short times of treatment (red line= 20 minutes, orange=50 minutes, yellow= 90 minutes) we can observe how at the beginning of the treatment the distance to the nucleolus seems to increase slightly. However, after 120 minutes of treatment (green line) the position of the RP gene appears to move closer to the nucleolus. The effect is maximal at 240 minutes after the addition of the drug (blue line).

A
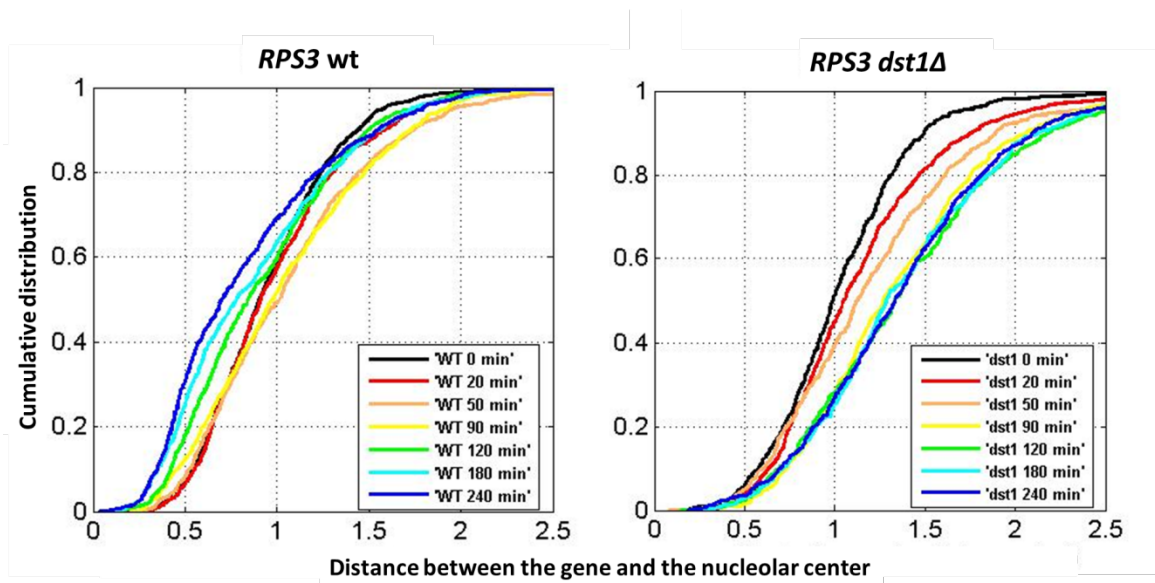
Figure 5 Effect of the addition of 6AU 100 µg/ml to the nuclear localisation of a RP gene in a wild type and an isogenic *dst1Δ* strains. Cumulative distribution curve of *RPS3* distance (x-axis in µm) to the nucleolus measured during a 2 hours treatment with 6AU 100 µg/ml in the wild type (left) and *dst1Δ* (right).

If we compare the high resolution probabilistic gene maps of *RPS3* and *RPL25* upon 6AU addition we can observe in the case of *RPL25* how the approximation to the nucleolus commences as soon as at 60 minutes and it reaches its maximal point at 120 minutes. After 120 minutes in 6AU it starts relocating to the original point (figure 6B). Despite their varying behaviour, the data obtained as well as the density images were consistently reproduced for both of the RP genes tested so we can confidently affirm that RP genes are temporally located closed to the nucleolus during transcriptional stress situations in a wild type background.

A

RPS3 WT

T=0′   T=15′   T=40′   T=90′   T=120′   T=180′   T=240′

B

RPL25 WT

T=0′   T=15′   T=40′   T=60′   T=90′   T=120′   T=180′   T=240′
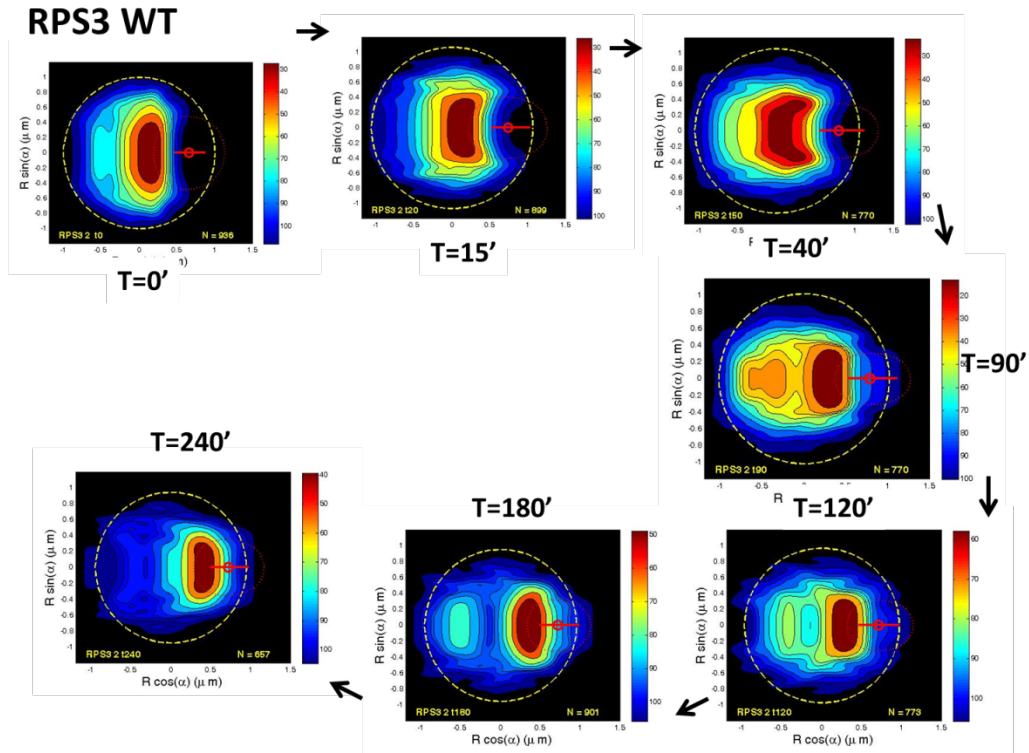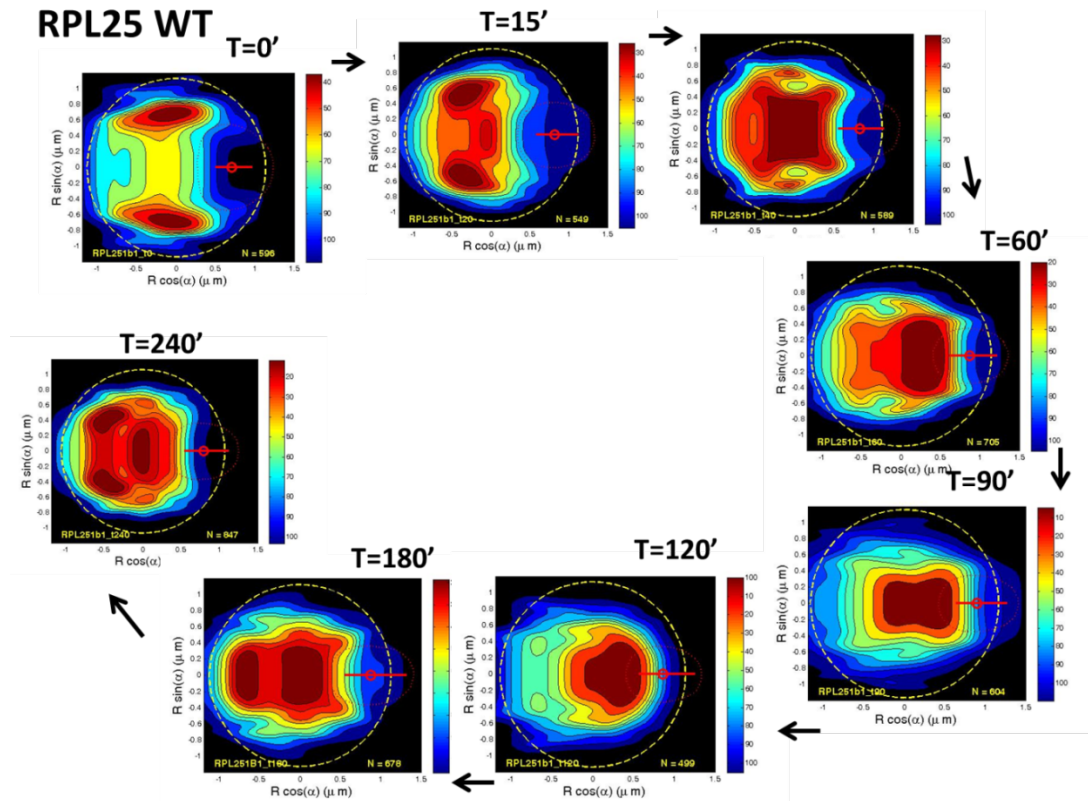
Figure 6 A High resolution probabilistic gene-maps for *RPS3* localisation and *RPL25* (**B**). The probability density of gene position is plotted relative to median (in the analised cell population) nuclear envelope and nucleolus.

3.1.4.2 Effect of 6AU in the nuclear localization of *RPS3* and *RPL25* in a *dst1Δ* strain

After confirming that RP genes' positioning in the nucleus changes in response to 6AU treatment we decided to analyse a *dst1Δ* strain in order to check if the observed change in the nuclear localisation of these RP genes was dependent on TFIIS. We had previously demonstrated that TFIIS plays an important role in the maintenance of the level of active RNA polymerases on RP genes. As these genes have shown a great sensitivity to a treatment with 6AU in the absence of TFIIS, we decided to study their location inside the nucleus of *dst1Δ* cells after the addition of 6AU 100µg/ml. The construction of the *dst1Δ* strains is described in Materials and Methods. Contrary to what could be observed in the wild type strain, in the *dst1Δ* background the genes did not approach to the nucleolus. In figure 5B we can see how the *RPS3* gene moved away from the nucleolus in *dst1Δ* until a distance that remains stable after 120 minutes of treatment.

In figure 7 we compare the behaviour of one of *RPL25* in wild type and *dst1Δ* cells at 15 and 90 minutes after the addition of 6AU. It was at these times that we could detect more clearly the effect of 6AU on RP genes transcription. We can appreciate that there is a change in the location of the *RPL25* gene in the wild type background at 90 minutes after the addition of the drug. It is especially evident when we observe the gene density maps (figure 7B). In *dst1Δ* we did not observe this movement towards the nucleolus, but in the opposite direction (figures 7A and 7B). Leaving aside the difference between the two RP genes tested these results demonstrate that RP genes undergo a displacement towards the nucleolus under conditions of transcriptional stress and that this change in their position depends on TFIIS.
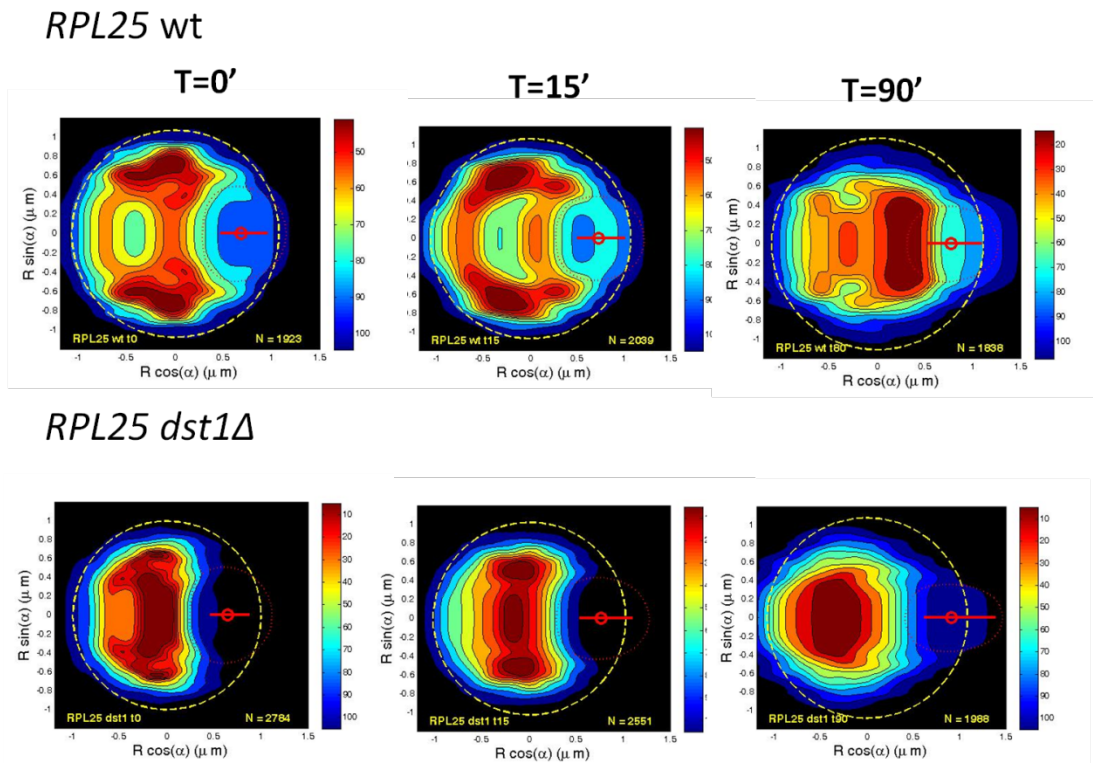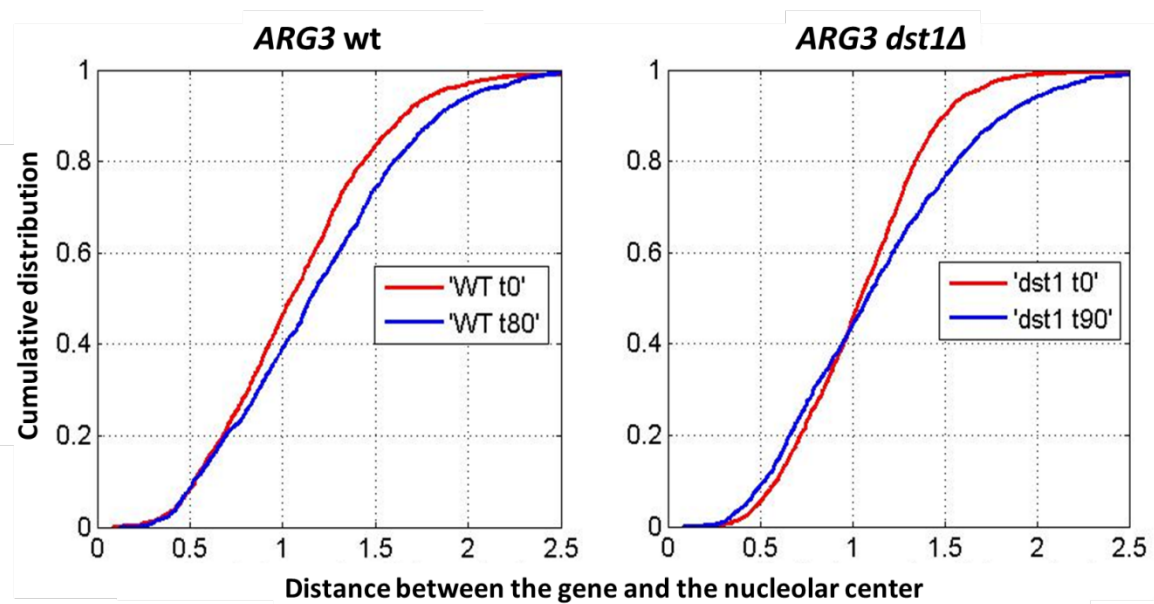
A
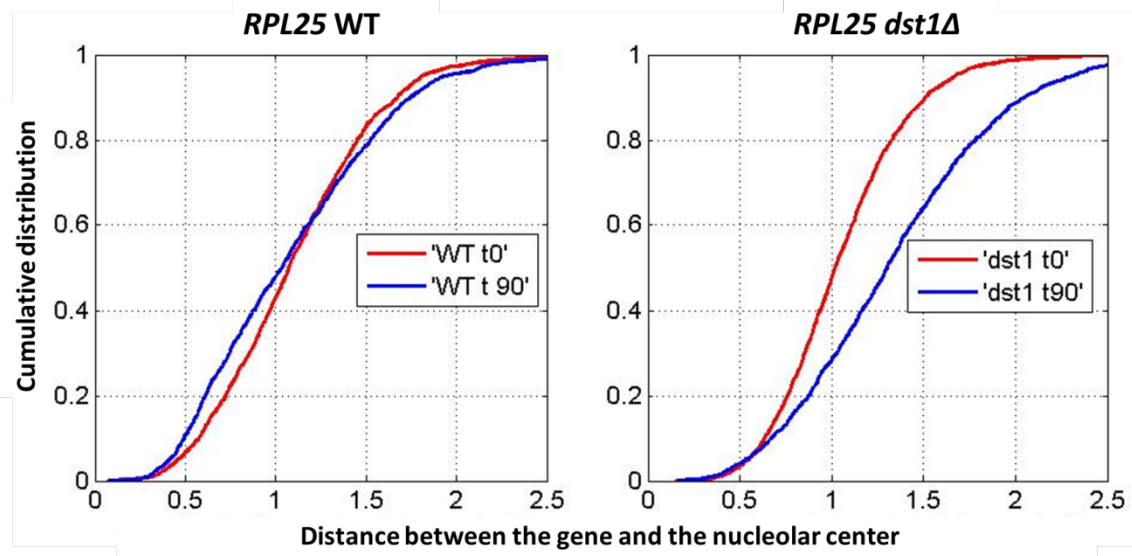
B

Figure 7 A Effect of the addition of 6AU 100 µg/ml to the nuclear localisation of the RP gene *RPL25* in a *dst1Δ* strain isogenic to the wild type. Cumulative distribution curve of *RPL25* distance (x-axis in µm) to the nucleolus measured during a 90 minutes treatment with 6AU 100 µg/ml. **B** High resolution probabilistic gene-maps for *RPL25* localisation with comparison between the wild type and the isogenic *dst1Δ* strains.

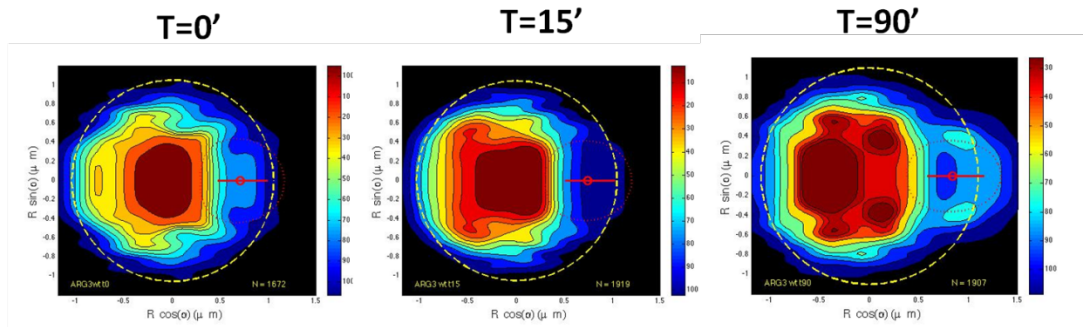### 3.1.4.3 Nuclear localization of *ARG3*, a non-RP gene.

Considering that our previous results show that RP genes move to the nucleolus as a response to nuclear stress and that this change is dependent on TFIIS the next step was to confirm that this change is exclusive of RP genes. In order to do so we decided to test a non-RP gene in both wild type and *dst1Δ* backgrounds. We chose the gene *ARG3* which encodes the enzyme ornithine carbamoyltransferase, which is not related to any ribosome biogenesis process and which should not be affected in the same manner than RP genes during a treatment with 6AU. This non-RP gene is located in chromosome X, and it is separated from any other RP coding gene by at least 100 Kb. We considered important to choose an isolated non-RP so we could rule out that any change in its position could be due to a dragging effect due to a close RP gene that was being relocated. After carrying out the same experimental procedure followed with the RP genes we could observe that the gene *ARG3* did not behave in the same manner than the RP genes upon 6AU addition (figure 8). In this case, when we analysed the wild type strain we observed that this gene did not move closer to the nucleolus, not even after long periods of 6AU treatment. As it was expected, the position of *ARG3* did not move closer to the nucleolus in a *dst1Δ* background either. This result confirms that the TFIIS-dependent effect on RP localisation after the induction of nuclear stress by the addition of NTP depleting drugs such as 6AU is not a general phenomenon of transcribed genes.
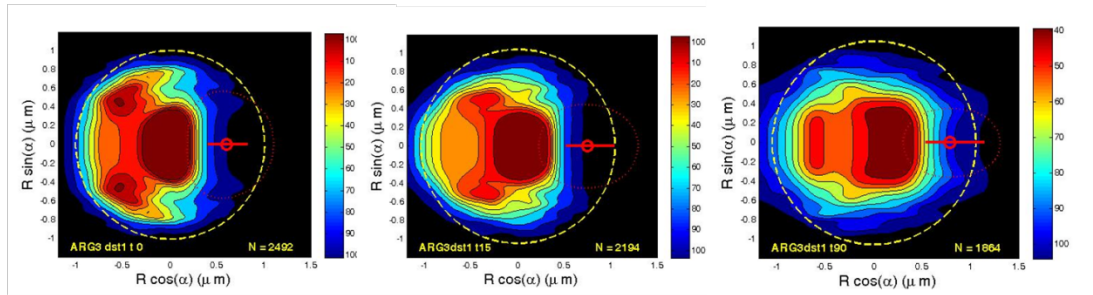
A

B

ARG3 wt

ARG3 dst1Δ

Figure 8 A Effect of the addition of 6AU 100 µg/ml to the nuclear localisation of a non-RP gene, *ARG3* in a *dst1Δ* strain isogenic to the wild type. Cumulative distribution curve of *ARG3* distance (x-axis in µm) to the nucleolus measured during a 90 minutes treatment with 6AU 100 µg/ml. **B** High resolution probabilistic gene-maps for *ARG3* localisation with comparison between the wild type and the isogenic *dst1Δ* strains.

3.1.5 Effect of 6AU on the geometry of the cell.

It could be argued that the nuclear stress caused by the addition of 6AU may be affecting the cell geometry. Under these circumstances the observed change in the localisation of RP genes could be due to an indirect effect. In order to rule out this possibility we also measured some parameters that could be affected in nuclear stress conditions. Changes in the nuclear and nucleolar volumes can affect the relative distances inside the nucleus. In this case, the localisation of a single gene would appear to vary. To obtain reliable data we used two of the strains previously analysed: the *RPL25* and the *ARG3* labeled strains in wild type and *dst1Δ* backgrounds. When we obtained the data that corresponded to nuclear and nucleolar volume values we could observe that the addition of the drug was effectively causing a change in the geometry of the cell. In figure 9 we have represented the change in nucleolar volume after

6AU addition. After 90 minutes upon 6AU addition the volume of the nucleoli have decreased in all the strains checked. This involves that the detected movement of RP genes towards the nucleolus requires an active relocation of them. We also found a reduction in the nucleolar volume of *dst1Δ* cells upon 6AU treatment. This might indicate that the increased distance between RP genes and the nucleolus detected in *dst1Δ* after the addition of 6AU would be rather a passive effect. According to this, the absence of TFIIS would impair the movement of RP genes towards the nucleolus upon 6AU treatment, but would not provoke an active relocation of RP genes away from this subnuclear compartment.
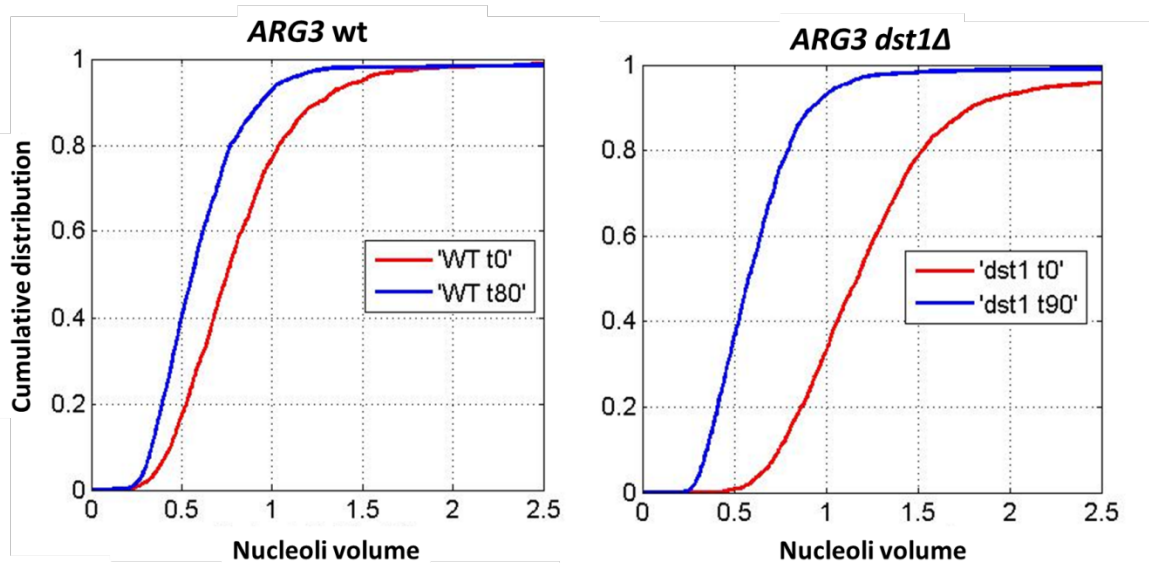
A
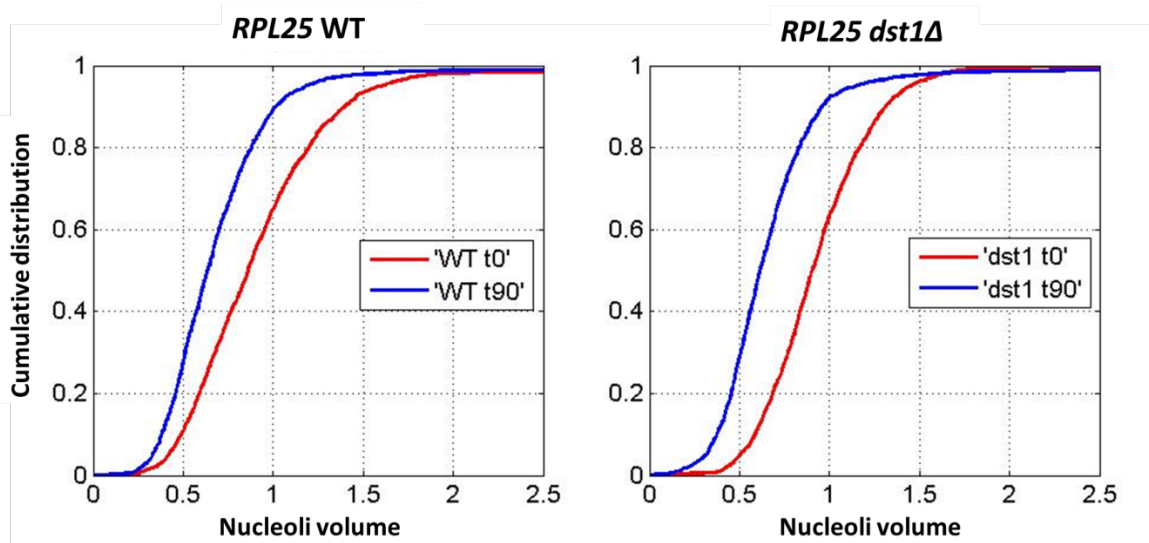
B

Figure 9 A Effect of the addition of 6AU 100 µg/ml to the nuclear geommetry of the cell in a wild type and an isogenic *dst1Δ* strain. Cumulative distribution curve of the changes in nucleolar volume in the strains used in the experiments *RPL25* (**A**) and *ARG3* (**B**). In the x-axis is represented the change in nucleolar volume during a 90 minute treatment with the drug.

Chapter 2

RNA polymerase II backtracking across the genome

and its contribution to gene expression

3.2.1 *sfp1Δ* is an optimal tool to investigate RNA pol II backtracking

Fernando Gómez-Herreros isolated in Chávez's lab several mutations able to suppress the sensitivity to NTP-depleting drugs exhibited by *dst1Δ* (F. Gómez-Herreros, Tesis Doctoral, Universidad de Sevilla). Most of these mutations were related to ribosome biogenesis and two of them (*sfp1Δ* and *sch9Δ*) affected regulators of RP and RiBi genes (Gómez-Herreros et al, 2012). In this chapter, we first characterised the mechanism of the suppression by *sfp1Δ*, and found that it is an optimal tool to investigate RNA pol II backtracking.

3.2.1.1 *sfp1Δ* suppresses *dst1Δ* sensitivity to NTP depleting drugs.

Gómez-Herreros' results suggested that *sfp1Δ* would be able to prevent the deleterious effects produced by 6AU on RP genes in the absence of TFIIS. In order to confirm this hypothesis, we analysed the transcriptional response of both RP and non-RP genes to 6AU in the *sfp1Δ* and *dst1Δsfp1Δ* backgrounds. Unlike the marked decrease detected in the RNA polymerase II occupancy of RP genes upon 6AU addition in the wild type, a reduction of only the 20% was observed in *sfp1Δ* (figure 10 A). Likewise, the RNA polymerase II decrease of the four RP genes in *dst1Δ* was absolutely abolished in the double mutant. Similar results were obtained with the run-on assay (figure 10 B). Consequently upon 6AU addition, the run-on/Rpb3 ratios of the RP genes in *sfp1Δ* and in *dst1Δsfp1Δ* did not undergo any significant variation that was comparable to those exhibited by the same genes in the wild type or in *dst1Δ*, (figure 10 C).
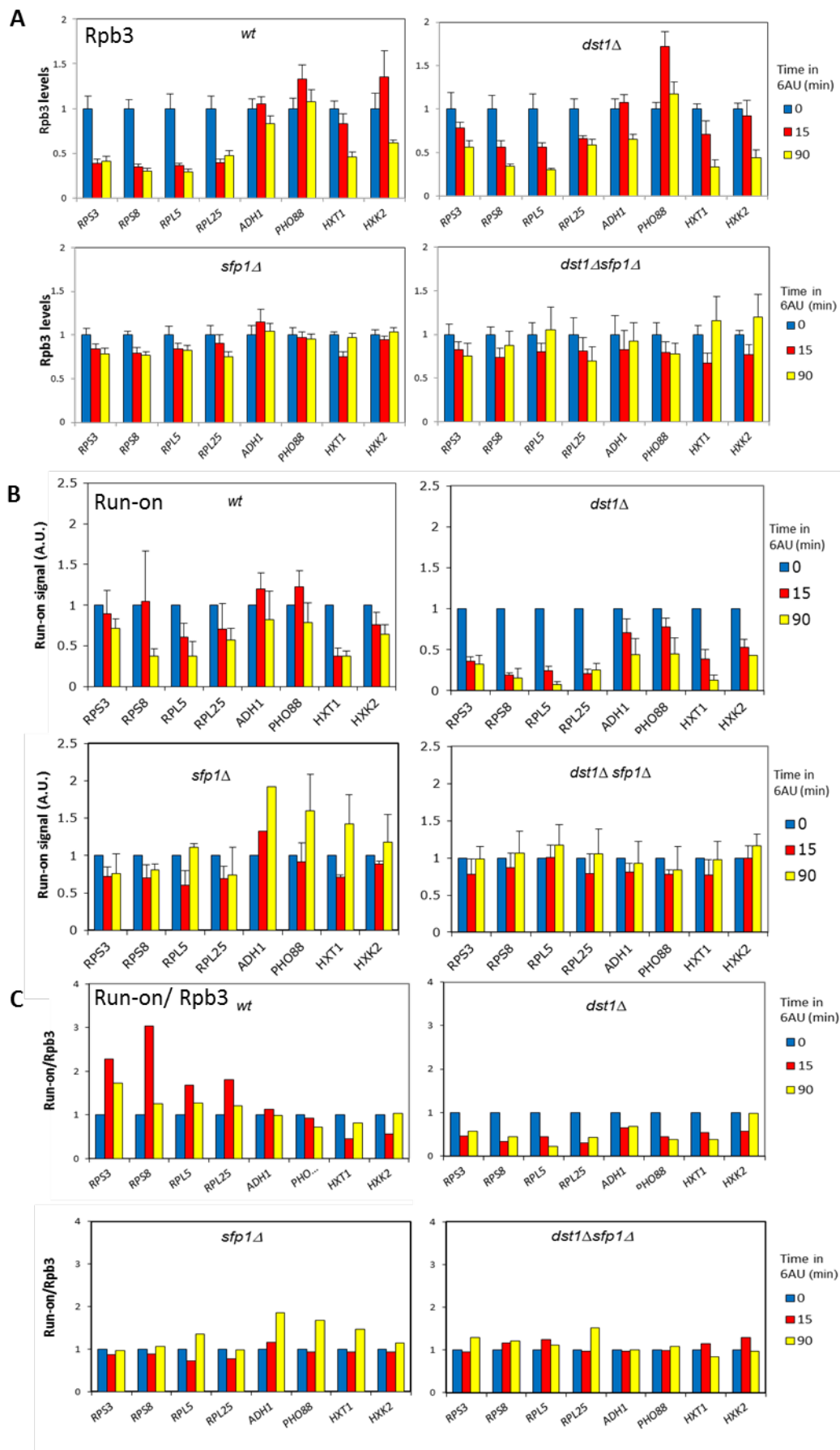
Figure 10 *sfp1Δ* suppresses the transcriptional phenotypes of *dst1Δ* in RP and non RP genes.**A** Average of Rpb3 IP signal for each gene in both strains and normalised to time 0. **B** RNA polymerase II activity as measured by transcriptional run-on on the indicated genes. **C** Variation in the specific activity of RNA polymerases sitting on the indicated genes expressed as the ratio between the variation in the transcriptional run-on signal and variation in the Rpb3 ChIP signal normalised to time 0. To facilitate the comprehension of the data, the information contained in figure $ was included in this figure again.

sfp1Δ did not eliminate all physiological effects of 6AU. For instance, the effect of 6AU on RNA polymerase I transcription was clearly visible on rDNA indicating that *sfp1Δ* should not prevent the effect of 6AU on NTP polls. (figure 11). We conclude that the transcriptional behaviour of RP genes changes in the absence of Sfp1, to such an extent that it enables them to remain active in the presence of 6AU, independently of TFIIS. This change might merely consist in a general down-regulation of the number of initiating RNA polymerase II molecules, as deduced from the low Rpb3 ChIP signals of the RP genes in *sfp1Δ* and *dst1Δsfp1Δ* (figure 12). However our results provide some clues to suggest that Sfp1's transcriptional role could go beyond merely regulating RP genes' RNA polymerase II initiation.
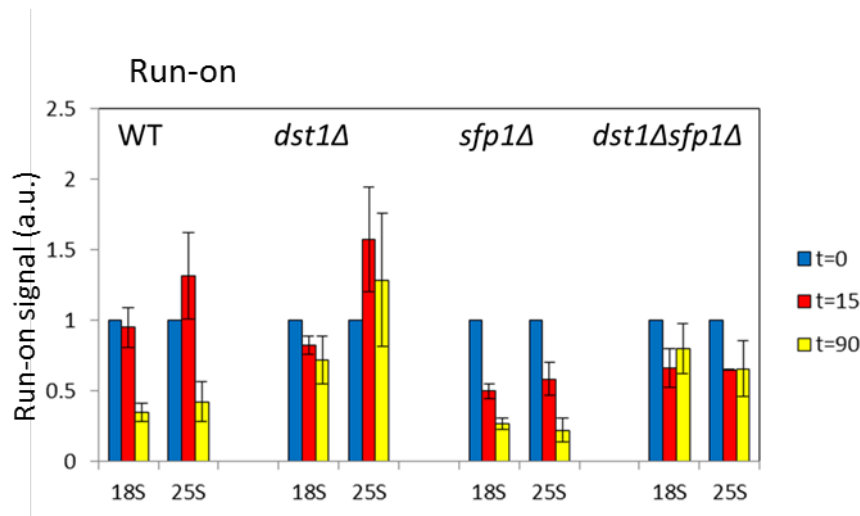

Figure 11 RNA polymerase I activity measured by run on in rDNA.

One intriguing finding is that the absence of Sfp1 not only abolishes the differential response of RP genes to 6AU, but also seems to modify non-RP transcriptional response to 6AU. Several pieces of data support this view. Firstly, non-RP genes also showed lower levels of RNA polymerase II in *sfp1Δ* and *dst1Δsfp1Δ* than in the wild type or in *dst1Δ,* even in the absence of 6AU (figure 12). No accumulation of RNA polymerase II at the 5'-end of non-RP genes was observed in either *sfp1Δ* or *dst1Δsfp1Δ* upon 6AU treatment (figure 12). Moreover,

no decrease in either RNA polymerase II occupancy or the run-on signal was observed for non-RP genes in *dst1Δsfp1Δ,* even after 90 minutes in the presence of 6AU (figure 10 A, B). Accordingly, the run-on/Rpb3 ratios of the non-RP genes in *dst1Δsfp1Δ* did not change upon 6AU addition (figure 10 C), which contrasts to their significant variation in *dst1Δ* (figure 10 C). Finally, the run-on signals and run-on/Rpb3 ratios of the non-RP genes in *sfp1Δ* increased after 90 minutes in the presence of 6AU (figure 10 B, C). All these data suggest a general effect of *sfp1Δ* on elongation by RNA polymerase II, which should be especially relevant for the transcription of RP genes. As we have previously shown that RNA pol II backtracking is particularly frequent in RP genes, we hypothesise that *sfp1Δ* prevents RNA pol II backtracking.
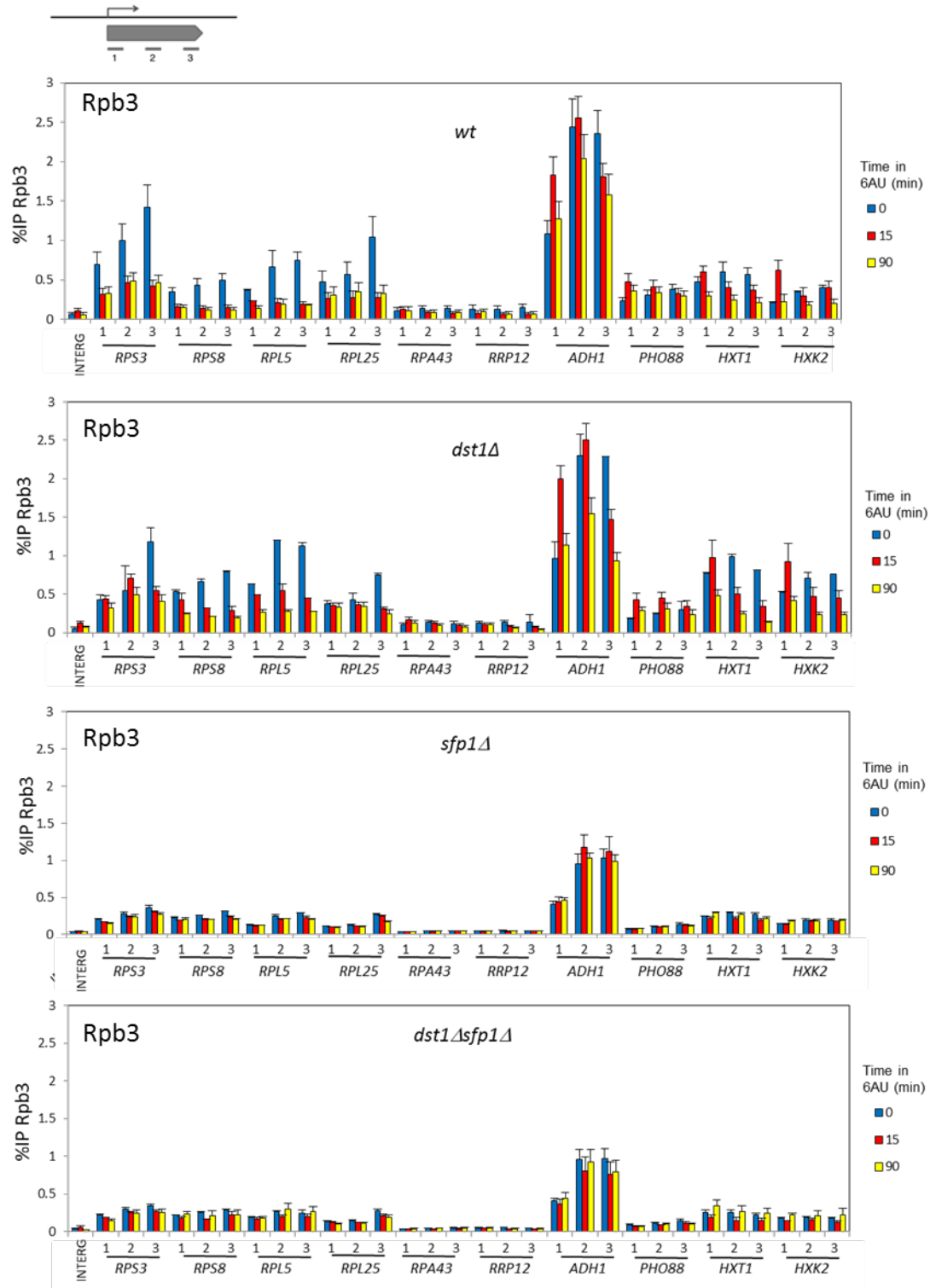
Figure 12 Variation in the levels of RNA polymerase II bound to the indicated genes caused by the addition of 6AU 100 μg/ml to *sfp1Δ* and *dst1Δsfp1Δ* cells. All the values represent the average of three independent experiments and three different amplicons distributed along the indicated genes.

3.2.1.2 *sfp1Δ* overcomes TFIIS recruitment to highly transcribed genes

As we previously demonstrated, *sfp1Δ* suppresses *dst1Δ* sensitivity to 6AU, a situation in which RNA polymerase II backtrack is more frequent. According to our working hypothesis, the mechanism of this suppression would be related to the effect of *sfp1Δ* in preventing RNA polymerase II's backtracking. This hypothesis predicts a significant decrease in the recruitment of TFIIS to transcribed genes in *sfp1Δ*. In order to test this, we performed ChIP experiments against C-terminal HA tagged TFIIS and Rpb3 (see Materials and Methods). We studied the distribution of TFIIS and Rpb3 by qPCR along RP and non-RP genes. For the qPCR we made use of the same amplicons that we had previously analysed in other experiments. We found that in *sfp1Δ* TFIIS is not recruited to any group of genes, while in the wild type strain the distribution profile of TFIIS occupancy was similar to that of the RNA polymerase II (figure 13). This result supports our hypothesis that Sfp1 either directly or indirectly, is playing a role in RNA polymerase II transcription elongation not described until now. This role would favour RNA polymerase II backtracking in such a way that the absence of Sfp1 allows the cell to overcome the defect caused by the absence of TFIIS.
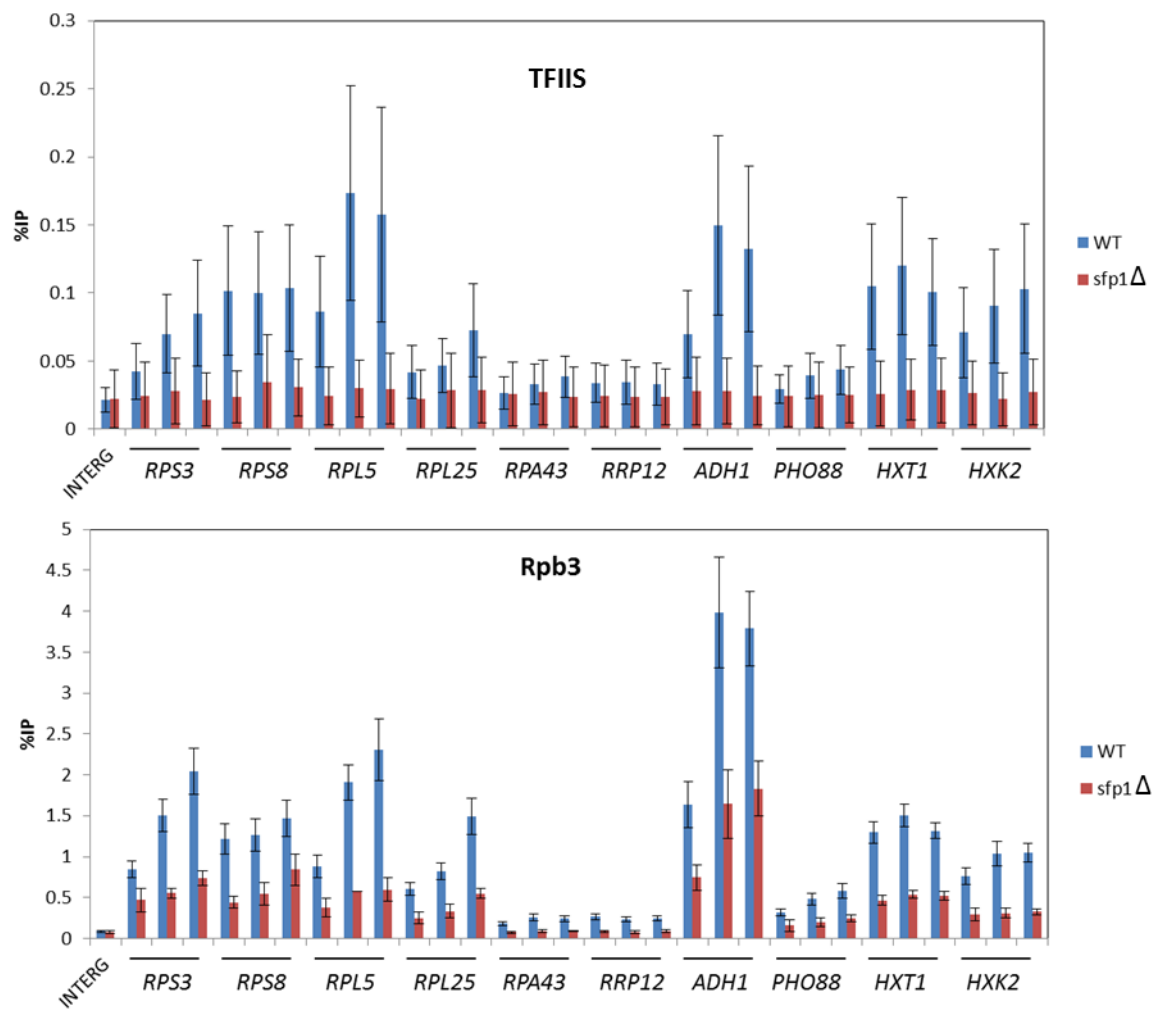
Figure 13 Effect of *sfp1Δ* onTFIIS recruitment. Variation in the levels of HA-TFIIS and RNA polymerase II subunit Rpb3 bound to the indicated genes in wild type and *sfp1Δ* cells. All the values represent the average of three independent experiments and three different amplicons distributed along the indicated genes.

3.2.1.3   Suppression of *dst1Δ* defect in Ser2 phosphorylation by *sfp1Δ*

The phosphorylation of the CTD residues can be used as a marker of RNA polymerase II activity. The CTD is hypo-phosphorylated when the polymerase binds to the promoter. When transcription initiates occurs the phosphorylation of the Ser5 residues that is higher during transcription initiation and starts decreasing when the phosphorylation of Ser2 increases, marking the phase of transcription elongation (see the introduction). We were interested in determine whether *sfp1Δ* or *dst1Δ* affected in some manner the dynamics of Ser2 phosphorylation. We checked the levels of Ser2P with respect to Rpb3 by chromatin immunoprecipitation and quantitative PCR. We got data from the wild type, and from *sfp1Δ*, *dst1Δ* and *dst1Δsfp1Δ* mutants, which we represented after calculating the Ser2P/Rpb3 ratios.
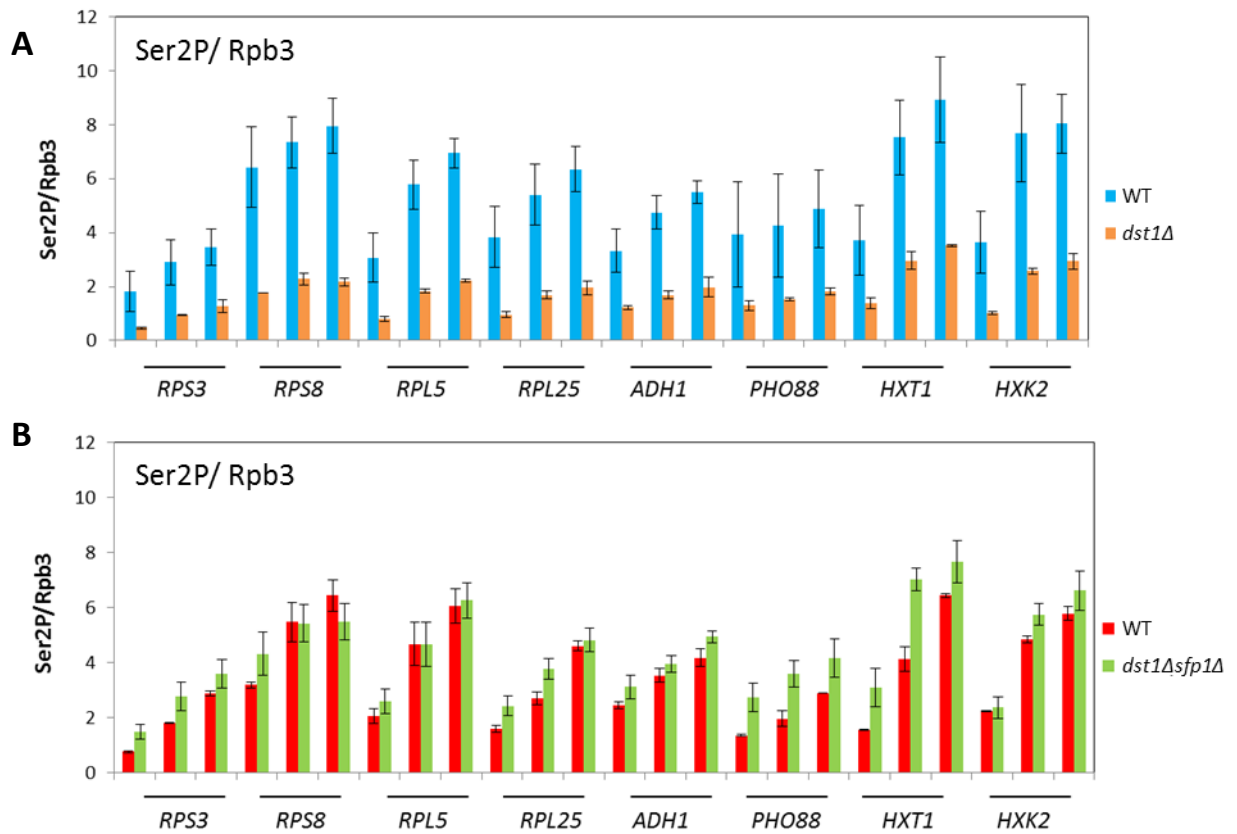
Figure 14 Effect of *dst1Δ* and *sfp1Δ* on Ser2 phosphorylation of Rpb1 CTD. Representation of Ser2P/Rpb3 ratios calculated from the ChIP values (% of input) against the phosphorylated form of Ser2 and Rpb3, analysed by qPCR. Comparison between the WT and a *dst1Δ* mutant (**A**) and a *sfp1Δ* and a *dst1Δsfp1Δ* double mutant (**B**). The error bars correspond to the standard deviation of the samples.

The absence of TFIIS provoked a severe decrease in Ser2 phosphorylation as compared with the wild type (figure 14 A). No general effect was detected in *sfp1Δ* (figure 14 A, B). However, in the *sfp1Δ* background, the absence of TFIIS did not produce a significant decrease in Ser2 phosphorylation (figure 14 B). So, the absence of Sfp1 rescue the effect of *dst1Δ* in a similar manner than it rescued the *dst1Δ* mutant sensitivity to 6AU, indicating that the decrease in the Ser2P/Rpb3 ratio shown by *dst1Δ* must be a consequence of unsolved backtracking events. It is interesting to note than the effect of *sfp1Δ* can be observed in all the genes tested, not only in RP genes. This supports our previous results where *sfp1Δ* appeared to have a general effect in all the genes transcribed by RNA polymerase II, either directly or indirectly.

3.2.2 RNA polymerase backtracking across the genome

In order to better understand RNA pol II backtracking we decided to measure its distribution and activity across the genome using two different genome-wide approaches. We carried out anti Rpb3 ChIP on chip experiments using Affymetrix® GeneChip *S.Cerevisiae* Tiling 1.0R custom arrays and a GRO (Genomic Run-On) assay. The latter was performed by Daniel Medina from Jose Enrique Pérez Ortín laboratory in the University of Valencia. These experiments were carried out as described in Materials and Methods. Contrary to the ChIP technique that allows the determination of total levels of polymerases on DNA regardless of their transcriptional state, the GRO assay allows the specific detection of those polymerases that are actively engaged in transcription. The combination of anti Rpb3 ChIP on chip and GRO allows us to determine the distribution of active and inactive RNA polymerase II across the genome.

3.2.2.1 Decreased RNA polymerase II occupancy of highly transcribed genes in *sfp1Δ*

In both, the Rpb3 ChIP and the GRO analysis we found a high correlation between the wild type and the *sfp1Δ* values (R^2 = 0.655 and 0.647 respectively). When we represent the average occupancy of Rpb3 in a wild type versus a *sfp1Δ* strain we can observe than those genes than present the highest Rpb3 occupancy in the wild type are the most affected in *sfp1Δ*, decreasing the occupancy of pol II in the mutant strain (figure 15 A). In contrast, the representation of GRO signal, which corresponds to active polymerases, shows a milder effect when we compare the wild type and *sfp1Δ* strains, 0.58 and 0.84 respectively (figure 15 B). This different effect of *sfp1Δ* on Rpb3 ChIP and GRO can be also observed when comparing the slopes of the equation of the tendency lines in the two graphical representations (figure 15 A, B). These results suggest that the transcriptional effects of *sfp1Δ* are mainly detected in highly transcribed genes and that they affect the total RNA polymerase II occupancy of these genes rather than the level of active (non-backtracked) RNA polymerases on them.
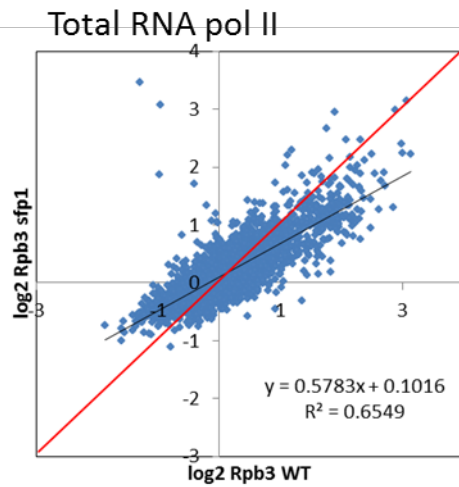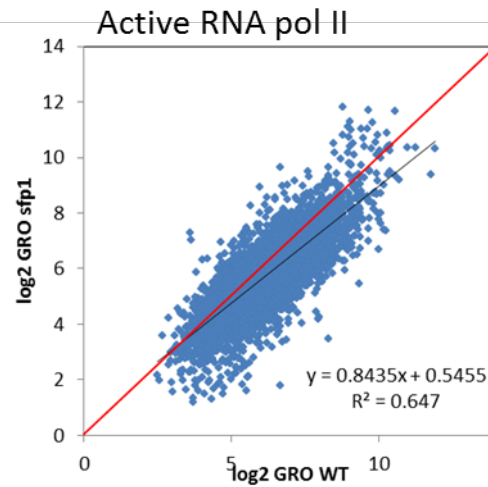
A

Total RNA pol II

$y = 0.5783x + 0.1016$
$R^2 = 0.6549$

B

Active RNA pol II

$y = 0.8435x + 0.5455$
$R^2 = 0.647$

Figure 15 Effect of *sfp1Δ* on RNA pol II occupancy and activity across the genome. **A** RNA polymerase II levels measured by anti Rpb3 ChIP on chip in a wild type versus a *sfp1Δ* strain. The values were obtained averaging the 5' and 3' values for each gene. **B** Pol II activity measured by GRO. The values were represented calculating the log2 of the data obtained after the analysis for both experiments.

We also represented both sets of data for each strain. We found a higher correlation between Rpb3 ChIP and GRO in the wild type (R^2=0.14) than in the mutant (R^2=0.06) (figure 16 A, B). We can see how RP genes that presented higher amounts of polymerases in the wild type showed a decreased number of the enzymes in *sfp1Δ,* although the level of GRO signal is not significantly affected in the mutant. This behaviour is not exclusive of RP genes, although this group of genes is the one that had been previously described to be controlled by Sfp1.
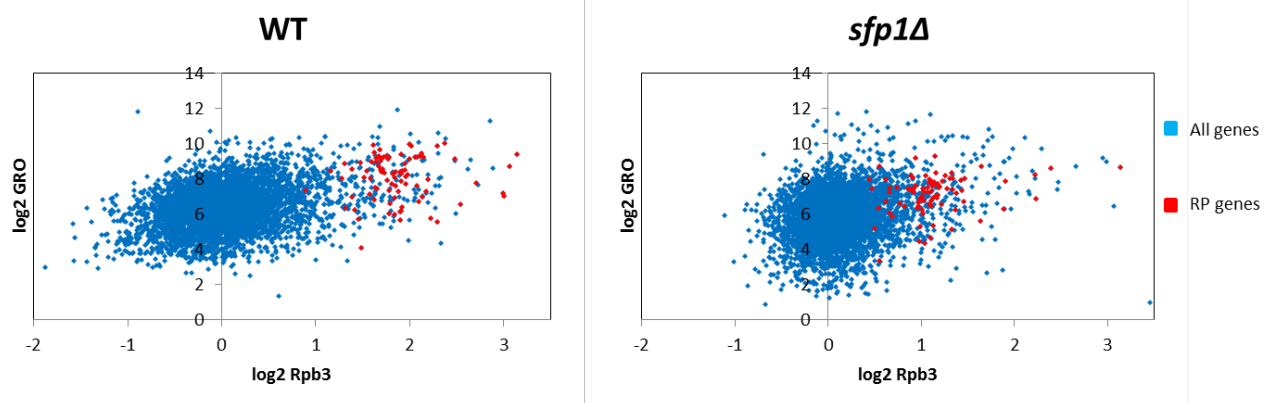
WT

sfp1Δ

Figure 16 Rpb3 ChIP versus GRO in a wild type and a *sfp1Δ* strain. The RP genes are highlighted in red.

3.2.4.2 *sfp1Δ* **exhibits decreased Rpb3/GRO ratios in highly occupied genes**

Those polymerases that are engaged in transcription and do not produce any run on signal are good candidates to be backtracked (Pelechano et al, 2009, Rodríguez-Gil et al, 2010).

We calculated the average Rpb3/GRO ratios for different groups of genes according to their transcriptional level: the four quartiles and the top decil according to the Rpb3 content (Q1, Q2, Q3, Q4 and D10) and the top decil according to the GRO signal (GRO10). We also calculated the average Rpb3/GRO ratio for the overlapping D10GRO10 group, and for those D10 genes not present in GRO10 (D10nonGRO10).

Considering the Rpb3/GRO ratio as an indicator of backtracking we found higher tendency to backtrack in those genes with highest Pol II occupancy (Q4, D10) (figure 17 A). We detected a significant negative impact of *sfp1Δ* on the Rpb3/GRO ratios of these genes (figure 17 A). In contrast the Rpb3/GRO ratios of GRO genes where extremely low in the wild type (lower than Q1), indicating that active transcriptional activity does not necessarily involve high occupancy. In this group of genes *sfp1Δ* did not produce lower Rpb3/GRO ratios, as expected (figure 17 A). Similarly, in D10GRO10, *sfp1Δ* did not produce a statistically significant impact (figure 17 A). The strongest effect of *sfp1Δ* on backtracking was detected in the group of genes with highest Rpb3/GRO ratios: D10nonGRO10 (figure 17 A). This differential effect of *sfp1Δ* on D10GRO10 and D10nonGRO10 was not due to a bias in the RNA pol II content of this two groups (figure 17 B). We conclude that the combination of Rpb3/GRO ratios and its modification by *sfp1Δ* are good tools to investigate RNA pol II backtracking genome-wide. Considering that genes bound by high levels of RNA pol II show the highest Rpb3/GRO ratios, and that their ratios are the most sensitive to *sfp1Δ,* we conclude that RNA pol II backtracking is particularly frequent in these highly occupied genes.

A

B
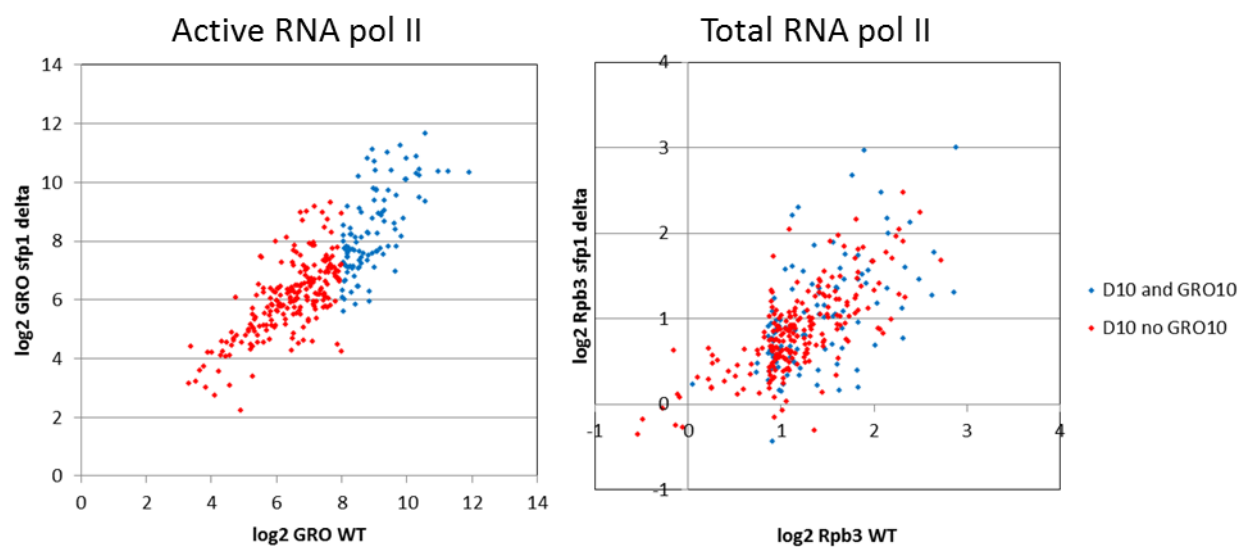
Active RNA pol II

Total RNA pol II
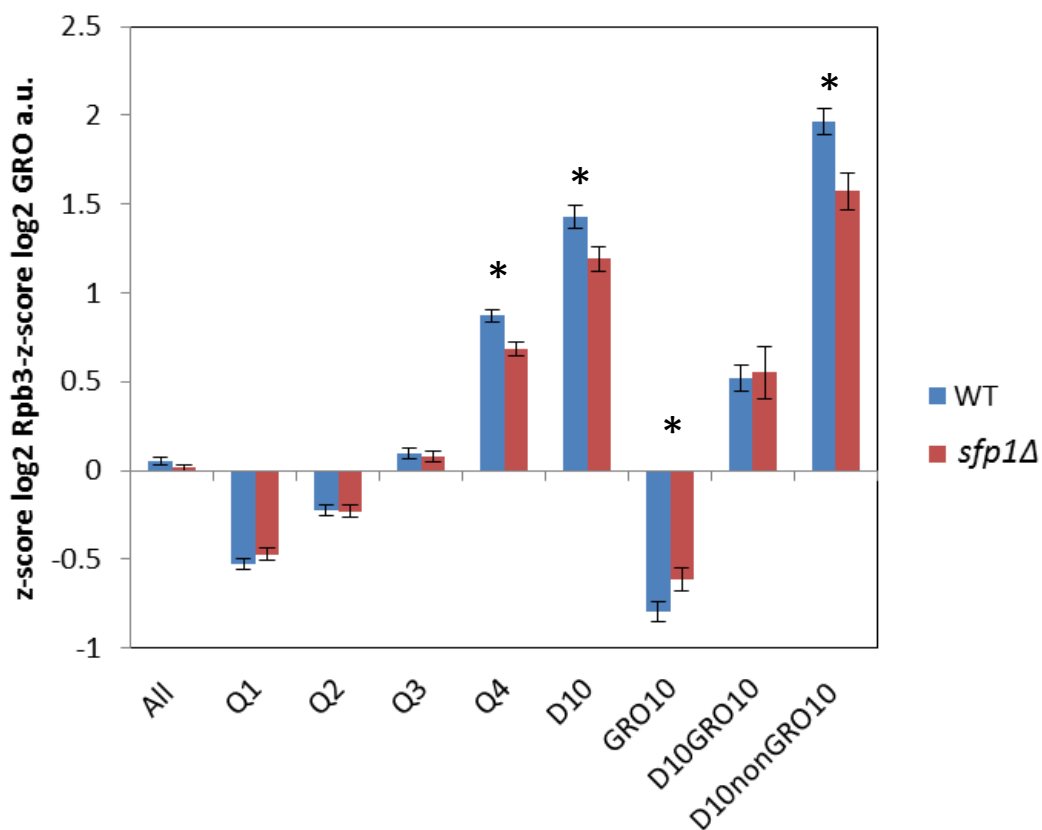
Figure 17 Incidence of RNA pol II backtracking among genes, depending on the level of transcription. **A** Rpb3/GRO ratios in a wild type versus a *sfp1Δ* strain. Cells were grown in glucose medium. To allow the comparison of both experiments we calculated the z-score log2 for all the data and represented the average for all the genes that belonged in each group. Q1, Q2, Q3, Q4 correspond to the four quartiles obtained after dividing the total of genes in four groups depending on their level of pol II. The stars mark those categories which medians are significantly different (p-value<0.05). **B** Representation of active polymerases and total polymerases in the groups of genes D10 and GRO10 (genes that present low backtracking) and D10 no GRO10 (genes that present high backtracking).

3.2.2.3. Highly occupied TATA-like genes are more prone to backtracking than TATA genes

Genes can be classified depending on their promoter architecture. Taking this into account we can divide genes in two distinct groups: TATA genes, which are those that present a canonical TATA box in their promoter sequence, and TATA-like genes, which consist of those genes that present a TATA-like element instead (Rhee and Pugh, 2012). Although RP genes are considered TATA-like, we analysed them separately, given their direct regulation by Sfp1 (Jorgensen et al, 2004). According to this classification, we decided to check whether the different proportion of backtracked RNA polymerase II that can be detected in different groups of genes could be related in any way to their type of promoter. In order to do so we divided the genes that we were studying in TATA and TATA-like. Only one third of the genes that belong to the D10 category belongs to the GRO10 as well (figure 18 A). Excluding RP genes, the D10GRO10 category is equally enriched in TATA and in TATA-like genes (figure 18 B). However, within D10, TATA-like genes tend to locate in D10nonGRO10 rather than in D10GRO10. Interestingly, within GRO10, TATA-like genes also tend to locate in GRO10nonD10 rather than in D10GRO10.

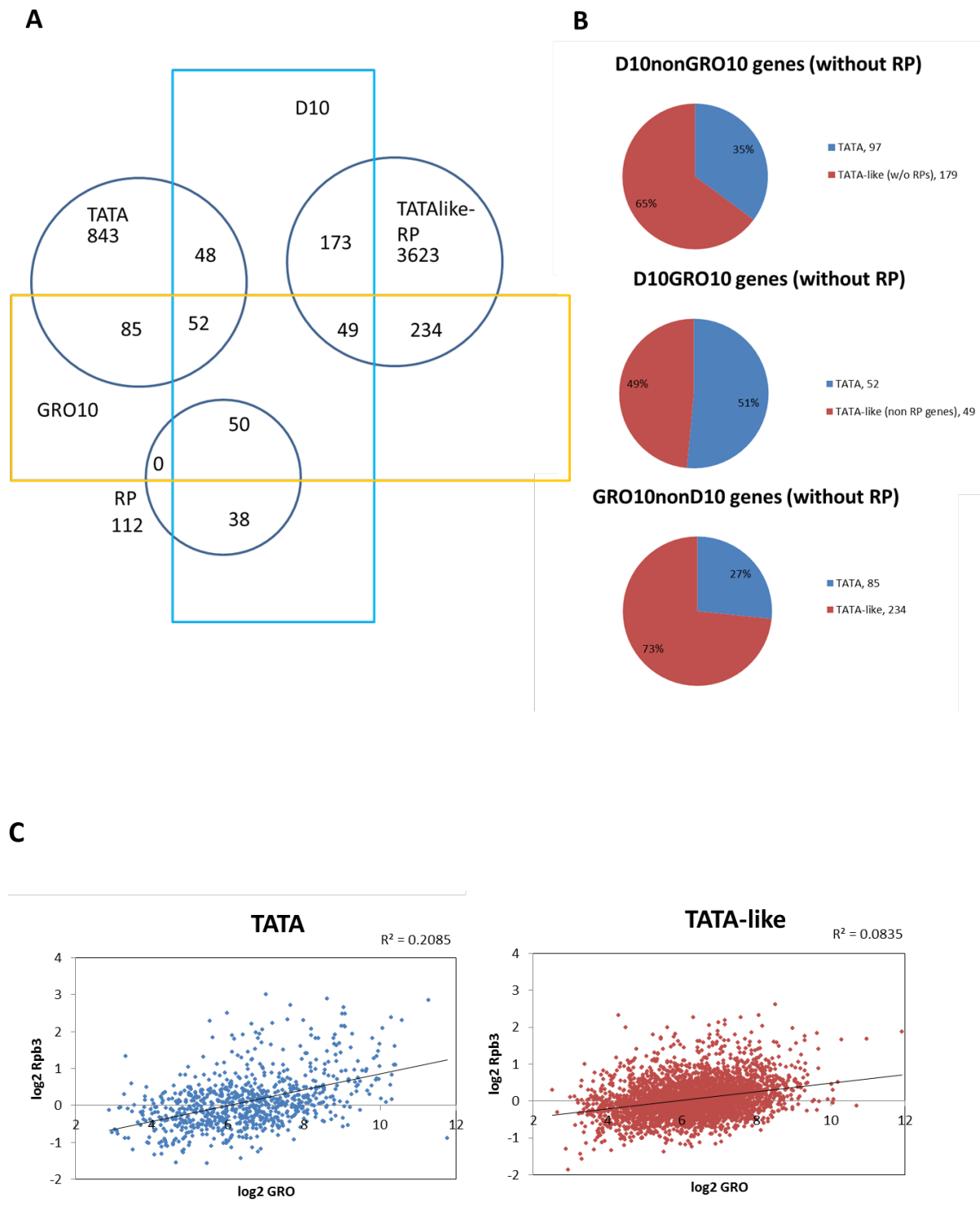**Figure 18** Quantitative distribution of TATA, TATA-like and RP genes in highly transcribed and highly occupied genes. **A** Number of genes that belong to each category used to filter the genomic data. **B** Percentage of genes in the D10 and GRO10 and D10 non GRO10 category that correspond to the TATA, TATA-like and RP genes groups. **C** Rpb3 versus GRO in TATA and TATA-like (non RP) genes in a wild type strain.

This lower correlation between RNA pol II occupancy and run-on signal in the TATA-like genes is detected even when all genes were analysed (figure 18 C). In spite of the higher absolute number of TATA-like genes, the Rpb3/GRO correlations was clearly lower in TATA-like (R^2= 0.2085) than in TATA genes (R^2= 0.0835).

3.2.2.4. Rpb3/GRO ratios of highly backtracking TATA-like genes are sensitive to *sfp1Δ*

As we can see in figure 17 A, the Rpb3/GRO ratios of the genes in the D10 non GRO10 category are sensitive to the lack of Sfp1. This group of genes are the more prone to backtracking. Given this bias in the promoter architecture of D10GRO10 and D10nonGRO10 genes, we analysed the Rpb3/GRO ratios in TATA and TATA-like genes. As expected, RP genes showed the highest Rpb3/GRO ratios in the wild type (figure 19 A) and they underwent a strong parallel decrease in GRO and Rpb3 ChIP in *sfp1Δ* (figure 19 B, C). When we excluded RP genes, we still found a decrease in Rpb3/GRO ratios in TATA-like genes, whereas we did not detect such a decrease in TATA genes (figure 19 A). Moreover, the Rpb3/GRO decrease in TATA-like (without RP) genes was due to a significant decrease in the Rpb3 signal (figure 19 B), without a parallel reduction in GRO (figure 19 C). No reduction, either in Rpb3 ChIP or in GRO was detected in TATA genes (figure 19 B, C).

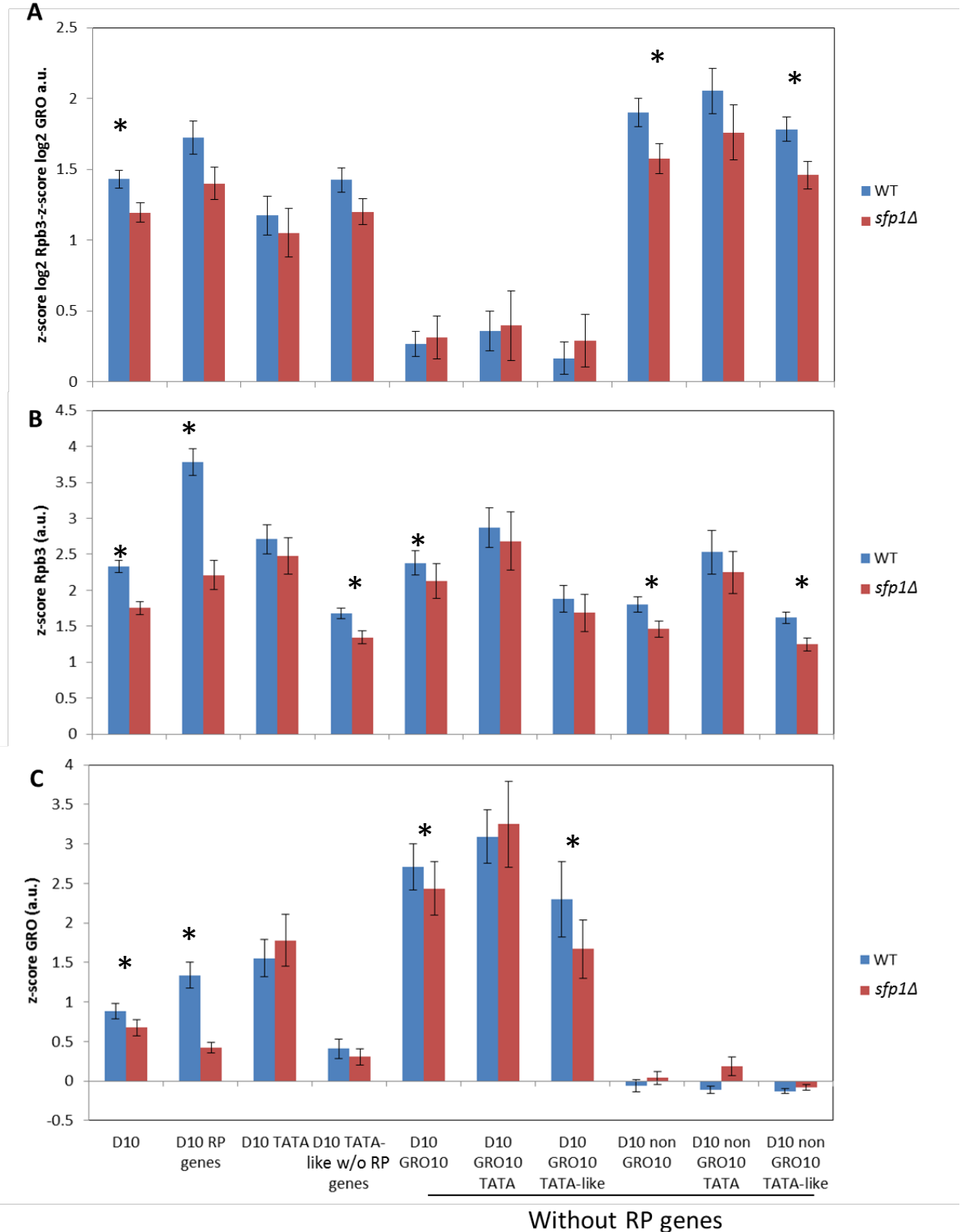**Figure 19** Effect of *sfp1Δ* on highly occupied genes. **A** Rpb3/GRO ratios in a wild type versus a *sfp1Δ* strain. Cells were grown in glucose medium (YPD). The z-score log2 for all the data was calculated to allow the comparison of the ChIP on chip and run-on experiments. The stars mark those categories which medians are significantly different (p-value<0.05). **B** Rpb3 levels. **C** GRO levels (RNA polymerase II activity).

The high Rpb3/GRO ratio of D10nonGRO10 genes was not a statistical artefact due to higher Rpb3 levels in this category. D10GRO10 genes showed even higher Rpb3 levels than D10nonGRO10 (figure 19B). We conclude that RNA pol II occupancy is not determining the distinction between D10GRO10 and D10nonGRO10.

Previous results have shown an influence of the carbon source (glucose or galactose) on backtracking (Pelechano et al, 2009). We wanted to know if the growth in galactose modifies in some way the proportion of active versus total polymerases and what would be the effect of *sfp1Δ* in this case. When we calculated the Rpb3/GRO ratios of those genes that present a high RNA polymerase II occupancy we found lower levels in galactose than in glucose (compare figure 17A and 20 A). In addition, we found a more general impact of *sfp1Δ* on Rpb3/GRO ratios in galactose than in glucose since even D10GRO10 genes showed significantly lower Rpb3/GRO ratios in *sfp1Δ* than in the wild type, when the cells were grown in galactose (figure 20 A).

A

Figure 20 Rpb3/GRO ratios in wild type and *sfp1Δ* cells grown in galactose. To allow the comparison of both experiments we calculated the z-score log2 for all the data and represented the average for all the genes that belonged in each group. The stars mark those categories which medians are significantly different (p-value<0.05).

This more general effect of *sfp1Δ* was also detected in TATA genes, which showed significantly lower Rpb3/GRO ratios in *sfp1Δ* than in the wild type when cells were grown in galactose (figure 21).

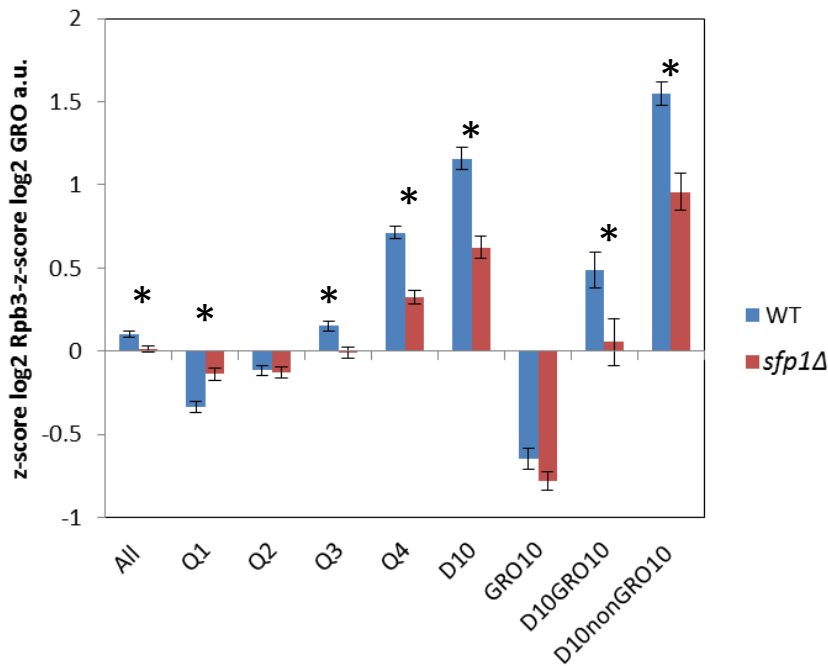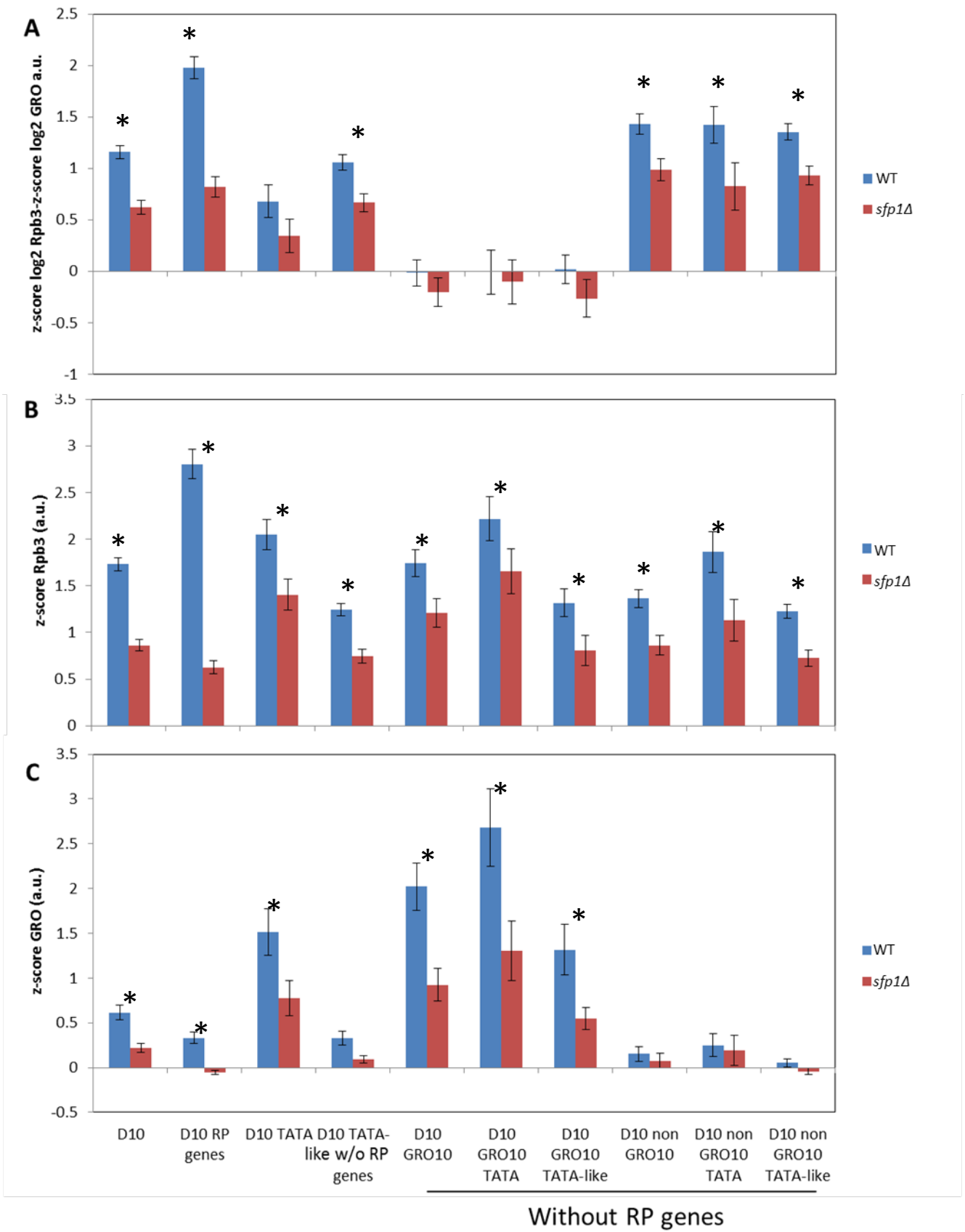**Figure 21** Effect of *sfp1Δ* on highly occupied genes of cells grown in galactose. **A** Ratios Rpb3/GRO in a wild type versus a *sfp1Δ* strain grown in galactose. **B** Rpb3 levels in a wild type versus a *sfp1Δ* strain grown in galactose. **C** GRO levels for the same groups of genes. In both experiments the data was represented calculating the z-score of the antilogarithmic data for each gene.

The levels of Rpb3 in all D10 categories decreased very significantly in galactose in the *sfp1Δ* strain with respect to the wild type (figure 21 B). However, the GRO levels also decrease in *sfp1Δ*, so the difference in the Rpb3/GRO cannot be exclusively due to a decrease in Rpb3 levels in the *sfp1Δ* mutant. We conclude that *sfp1Δ* produce a more general effect on backtracking when cells grow in galactose than in glucose.

3.2.3   RNA polymerase II configuration changes along the transcribed region and is extensively modified in *sfp1Δ*

The previous results showing very different Rpb3/GRO ratios among highly occupied genes led us to question how RNA pol II configuration changes among those genes, and in which ways the absence of Sfp1 influences this variation. To further investigate this, we decided to study the genomic distribution of the RNA polymerase II subunit Rpb4 in parallel with Rpb3 in a wild type and a *sfp1Δ* strains. Rpb4 is a non-essential subunit of the RNA polymerase II which forms a dissociable heterodimer with Rpb7. We expected that a major difference in the RNA pol II configuration would change the proportion between the ChIP signals of Rpb4 and Rpb3 (which forms part of the catalytic core of the enzyme). As we did for Rpb3 we amplified the samples obtained from a ChIP experiment against a version of Rpb4 tagged with the epitope Myc and sent them to the Multigenic Analysis Service of the University of Valencia where they were hybridised in Affymetrix custom microarrays. The resulting CEL files were analysed using the tiling array analysis software TAS and the R-language platform bioconductor. In this case we obtained the antilog$_2$ values of the data so we had positive numbers that permitted us to calculate the ratios between Rpb4 and Rpb3. We found several differences between the Rpb3 and Rpb4 profiles, which are more easily detectable in those genes with high RNA pol II levels (figure 22 A, Q4 and D10). Both Rpb3 and Rpb4 increased from the promoter region into the 5' transcribed region, but the profile of Rpb4 was increasing more progressively than Rpb3. Also in the 3' region, Rpb4 started to decrease before (more 5') than Rpb3. Finally, in the termination region, Rpb3 increased again up to a level clearly above the 5' region, whereas the Rpb4 increase in the termination region was much more subtle (figure 22 A). Accordingly to these differences, Rpb4/Rpb3 ratios decreased during

transcription initiation, showed a minimum at the 3' , increased again in the polyA signal and decreased again in the termination region (figure 22 B). We found different Rpb3 and Rpb4 profiles in the *sfp1Δ* strain too. Both profiles were less smooth along the 5' and 3' regions (figure 22 A). Also in *sfp1Δ* Rpb3 and Rpb4 profiles were not identical. The Rpb4 signal around the transcription start site was higher than the Rpb3 one, and the increase of Rpb4 in the termination region was less evident than the Rpb3 one (figure 22 A). The impact of *sfp1Δ* on RNA pol II configuration was clear when we calculated the Rpb4/Rpb3 ratios: no net change of this ratio was detected when we compared the initiation and the termination regions (figure 22 B). The general profile of Rpb4/Rpb3 ratio was totally different in *sfp1Δ*, compared to the wild type (figure 22 B).

A
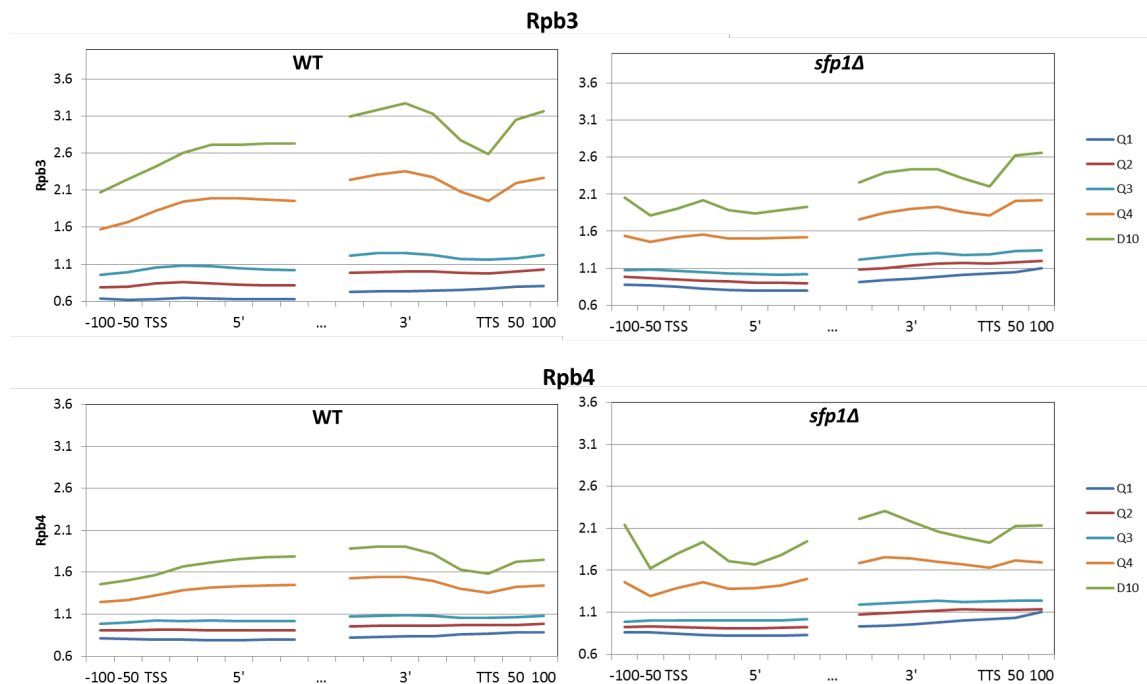
B

Figure 22 A *sfp1Δ* effect in RNA polymerase II configuration. The antilog$_2$ values of Rpb3 and Rpb4 were obtained from a ChIP on chip experiment against Rpb3 and Rpb4-Myc and the ratios between the values were calculated. **B** Ratios Rpb4/Rpb3 obtained from cultures grown in glucose. All the values were normalised to the -100 value in order to compensate the different levels of promoter-recruited polymerases in both strains.

We also obtained Rpb3 and Rpb4 profiles from cells grown in galactose as the carbon source. We found that the average profiles of Rpb3 were different in galactose than in glucose, something easier to detect in highly transcribed genes (compare figures 23 A and 22 A, Q4 and D10). In this case Rpb4 and Rpb3 profiles were more similar than they were in glucose, producing a less prominent change in Rpb4/Rpb3 ratio across the transcription unit (figure 23 B). In galactose, we also found different Rpb3 and Rpb4 profiles for *sfp1Δ* as compared with the wild type (compare figures 23 A and 22 A) and we also detected in these growing conditions a clear impact of *sfp1Δ* on the Rpb4/Rpb3 ratio across the metagene (figure 23 B).

Therefore, in two different metabolic conditions, we found a change of RNA pol II configuration during the transcription cycle, as reflected by the Rpb4 and Rpb3 ChIP signals, that was strongly influenced by *sfp1Δ.*
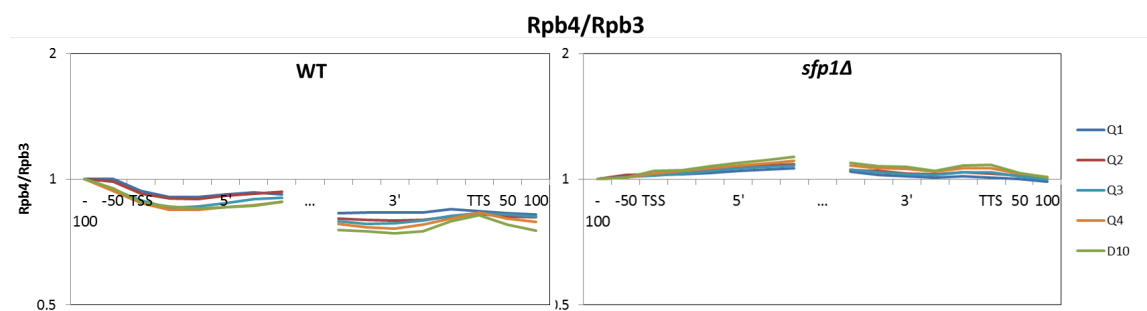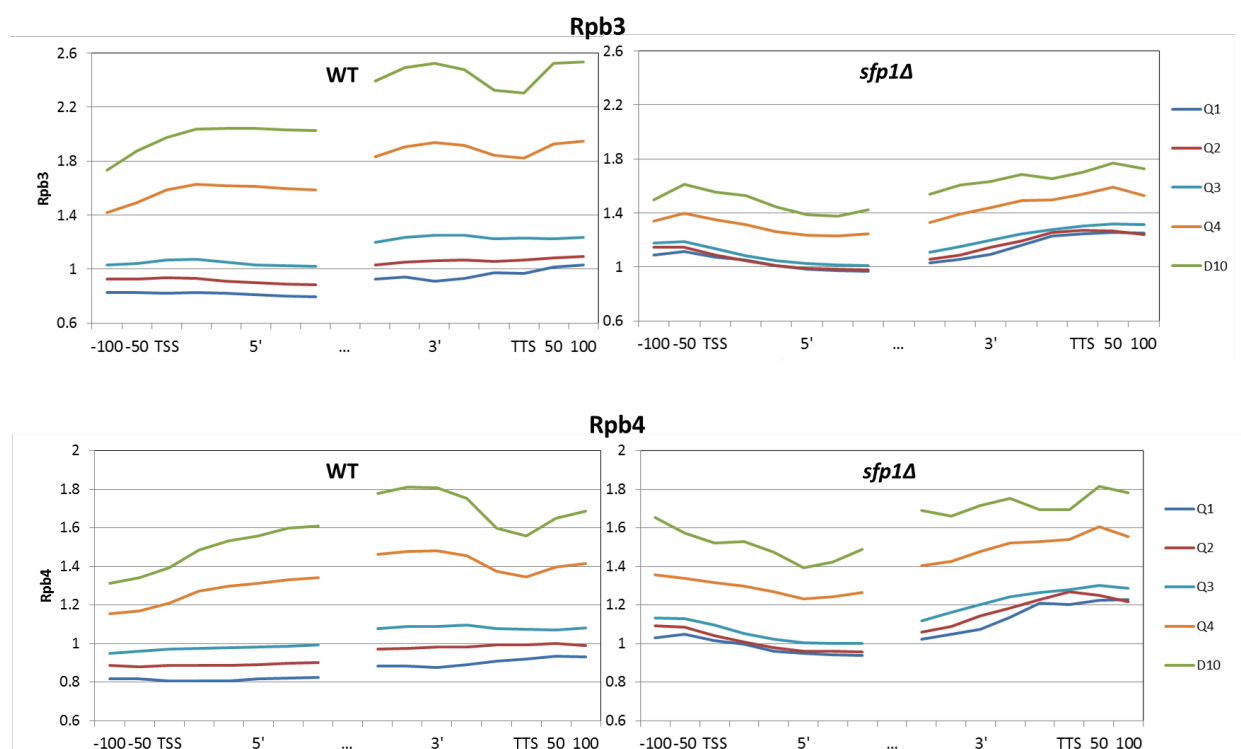
A

B
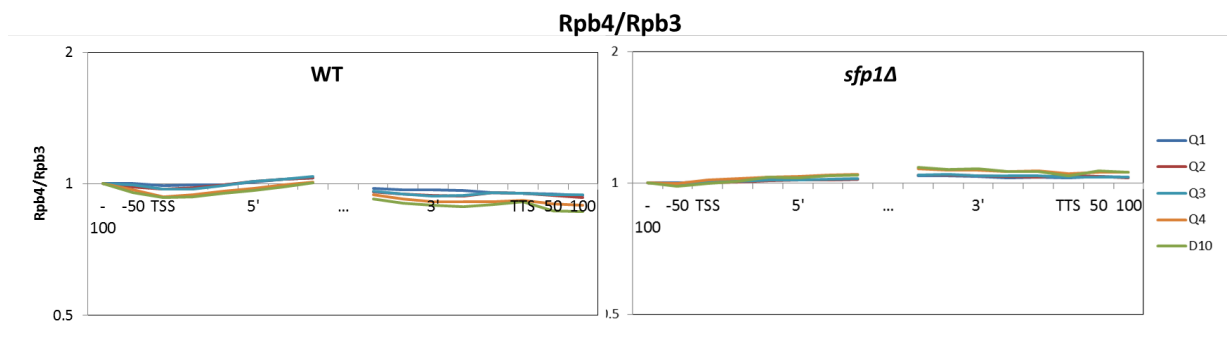
Figure 23 A *sfp1Δ* effect on RNA polymerase II configuration. The antilog$_2$ values of Rpb3 and Rpb4 were obtained from a ChIP on chip experiment against Rpb3 and Rpb4-Myc and the ratios between the values were calculated. **B** Ratios Rpb4/Rpb3 obtained from cultures grown in galactose. All the values were normalised to the -100 value in order to compensate the different levels of promoter-recruited polymerases in both strains.

3.2.4 Highly backtracking genes show particular RNA pol II profiles that are sensitive to *sfp1Δ*

We analysed the Rpb3 profiles and Rpb3/Rpb4 ratios of those genes showing a higher incidence of backtracking (D10nonGRO10) and those in which backtracking occurs less often (D10GRO10). We studied the Rpb3/Rpb4 ratios for each category and the individual profiles for Rpb3 (figure 24 A). We found clearly different Rpb3 profiles in the two groups of genes and important differences in the Rpb4/Rpb3 ratios as well. In the wild type there is a clear difference between the Rpb3 profiles of the D10nonGRO10 and D10GRO10 groups of genes. In the D10GRO10 group there is a fast increase of the levels of Rpb3 at the 5' end of the metagene, showing a step decrease at the polyA site and reaching a level at the transcription termination site similar to the level at the promoter. None of these characteristics can be observed in the D10nonGRO10 group, where the profile of Rpb3 shows a slow increasing tendency from the promoter to the transcription termination site, with a much less marked decrease at the TTS. With respect to the Rpb3/Rpb4 ratios, there is a decrease from 5' to 3' in D10nonGRO10, whereas in D10GRO10 this decrease is lower and is partially reversed in the termination region (figure 24 A). We detected similar profiles in the Rpb4/Rpb3 ratios of *sfp1Δ* in the two groups, although only the Rpb3 profiles of D10nonGRO10 genes were significantly affected by *sfp1Δ*. We conclude that those genes with a higher backtracking tendency show characteristic RNA pol II profiles and that the general change of RNA pol II configuration caused by *sfp1Δ* produces stronger effects in these genes.

As we had previously described the different behaviour of TATA and TATA-like genes with respect to *sfp1Δ* effect on RNA polymerase II backtracking we decided to check whether the RNA pol II profiles and Rpb4/Rpb3 ratios were also differently affected on both groups. We found that TATA genes behaved in average as the D10GRO10 genes whereas TATA-like did it as D10nonGRO10 genes (figure 24 B). In order to check whether backtracking or promoter type was the differentiating element, we compared TATA D10nonGRO10 to TATA D10GRO10 genes (figure 24 C), and TATA-like D10nonGRO10 to TATA-like D10GRO10 genes (figure 24 D). We found that the backtracking rate did not significantly affect the profiles of TATA genes or their response to *sfp1Δ,* whereas it was clearly influencing the profiles of TATA-like genes and their response to this mutation (figure 24 C-D). We also found that Rpb4/Rpb3 ratios were very close to 1 in sfp1Δ, in both 5' and 3', in all gene groups tested (figure 24). We conclude that backtracking provokes a particular RNA pol Ii profile in the context of TATA-like genes, and that the general changes in RNA pol II configuration caused by *sfp1Δ* modifies this backtracking behaviour.

In addition, we found a specific effect of *sfp1Δ* in the 3' profile of RNA pol II in TATA genes. In *sfp1Δ* there was a clean accumulation of RNA pol II in the termination region (figure 24 B). This effect was stronger in D10GRO10 than in D10nonGRO10 (figure 24 C), and was absent in TATA-like genes (figure 24 B, C).

A
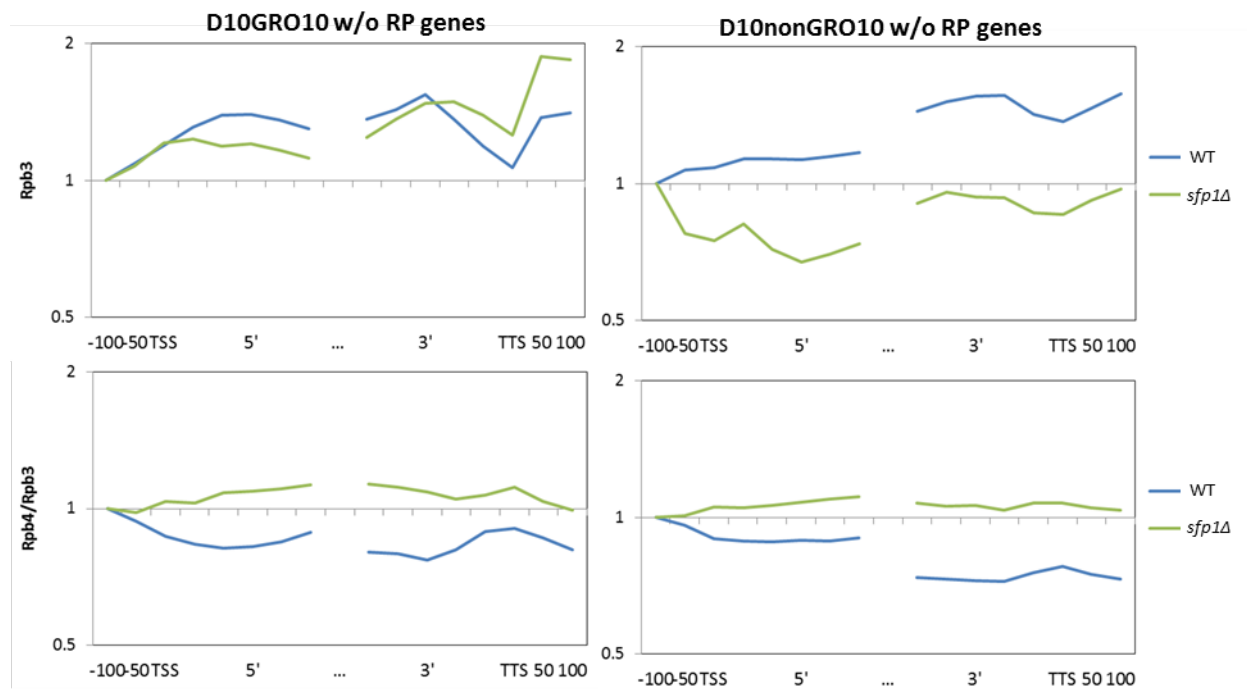
B
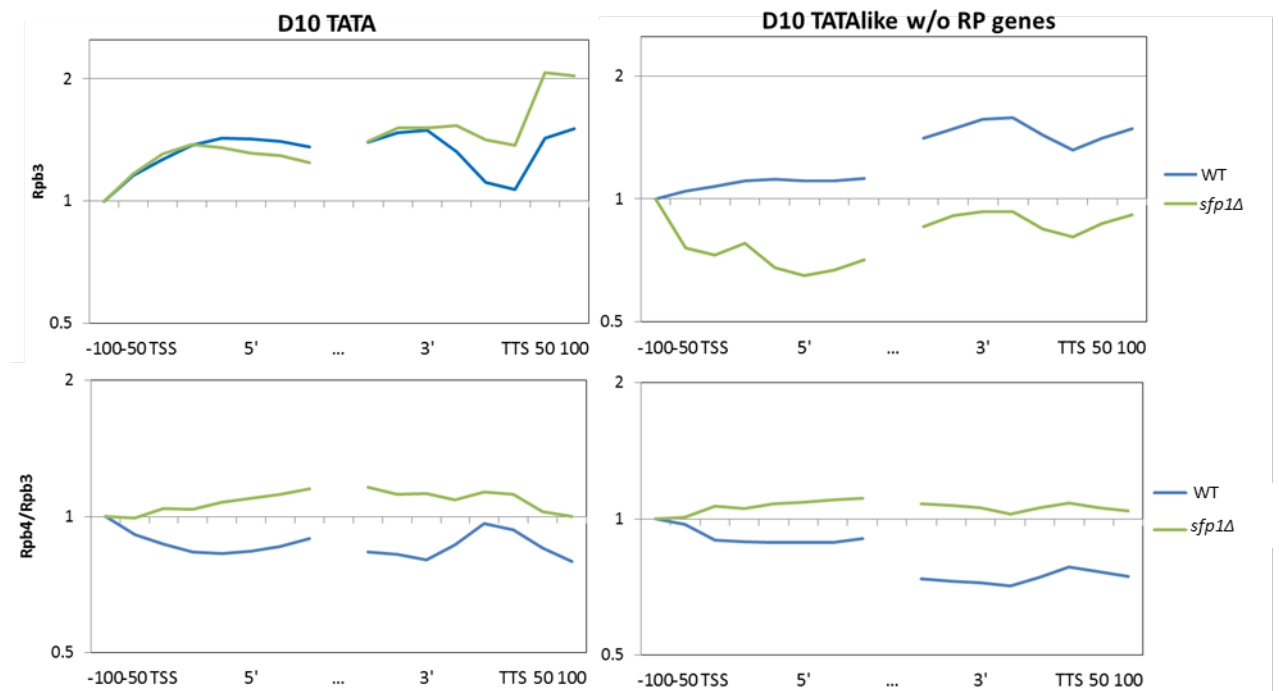
C
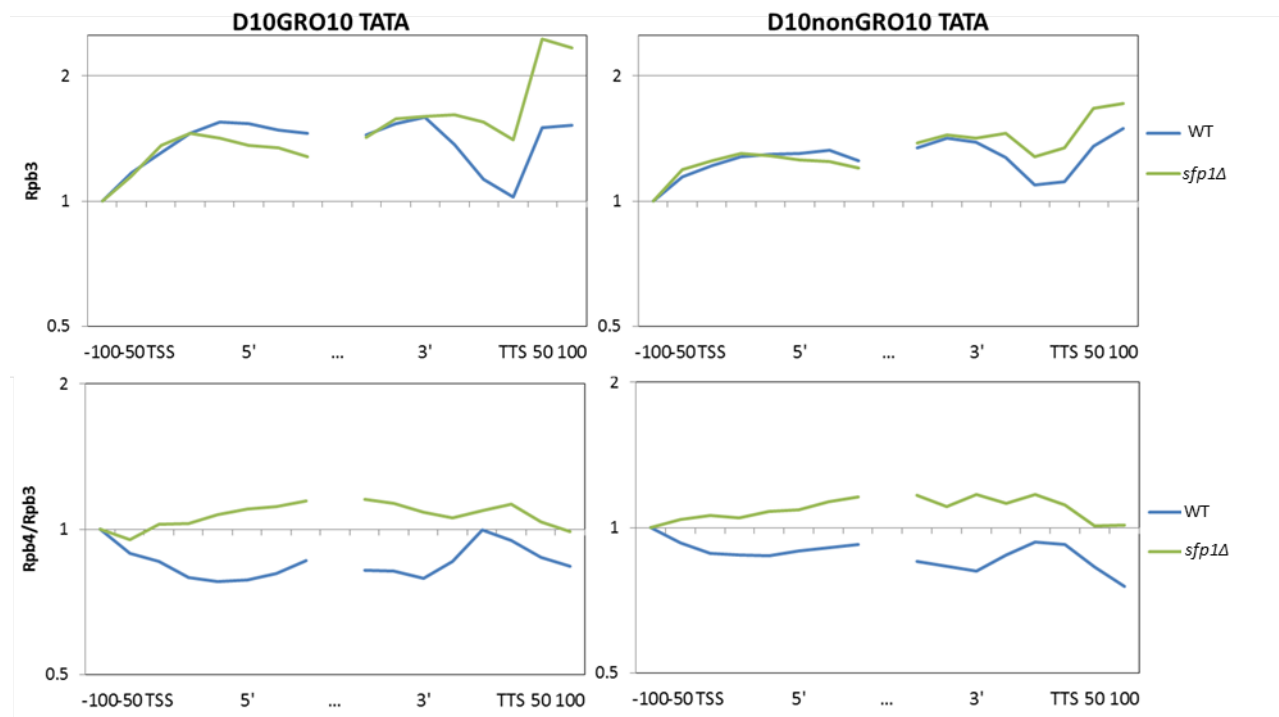
D
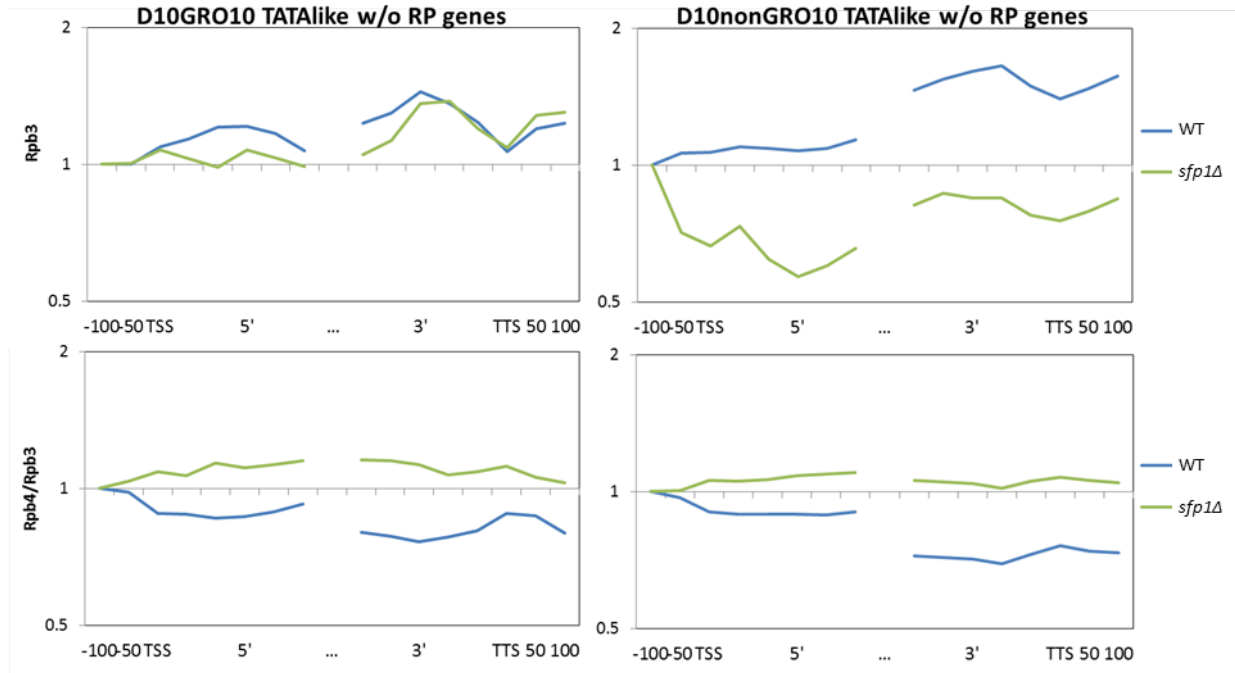
Figure 24 A Rpb3 profiles and Rpb4/Rpb3 ratios in glucose in the genes that belong to both groups, D10 and GRO10, genes that present low backtracking and D10 non GRO10, genes that present high backtracking levels. RP genes have been excluded from the list. **B** Highly transcribed genes (D10 category), divided in TATA and TATA-like genes. The RP genes have been excluded from the TATA-like category. **C** TATA genes divided in low (D10 and GRO10) and high (D10 non GRO10) backtrack categories. **D** Same as in (C) but for TATA-like genes without RP genes. All the values were normalised to the -100 value in order to compensate the different levels of initiating polymerases in both strains.

We made a similar analysis with the data of galactose-grown cells and found similar results. In this case the effect of *sfp1Δ* on RNa pol II profiles was much more general than in YPD (figure 25) although TATA-like genes were also more affected in the D10nonGRO10 subgroup than in D10GRO10 (figure 25 D). These results agree with the more general effect of *sfp1Δ* on backtracking detected in Rpb3/GRO ratios in galactose (figure 21).

A
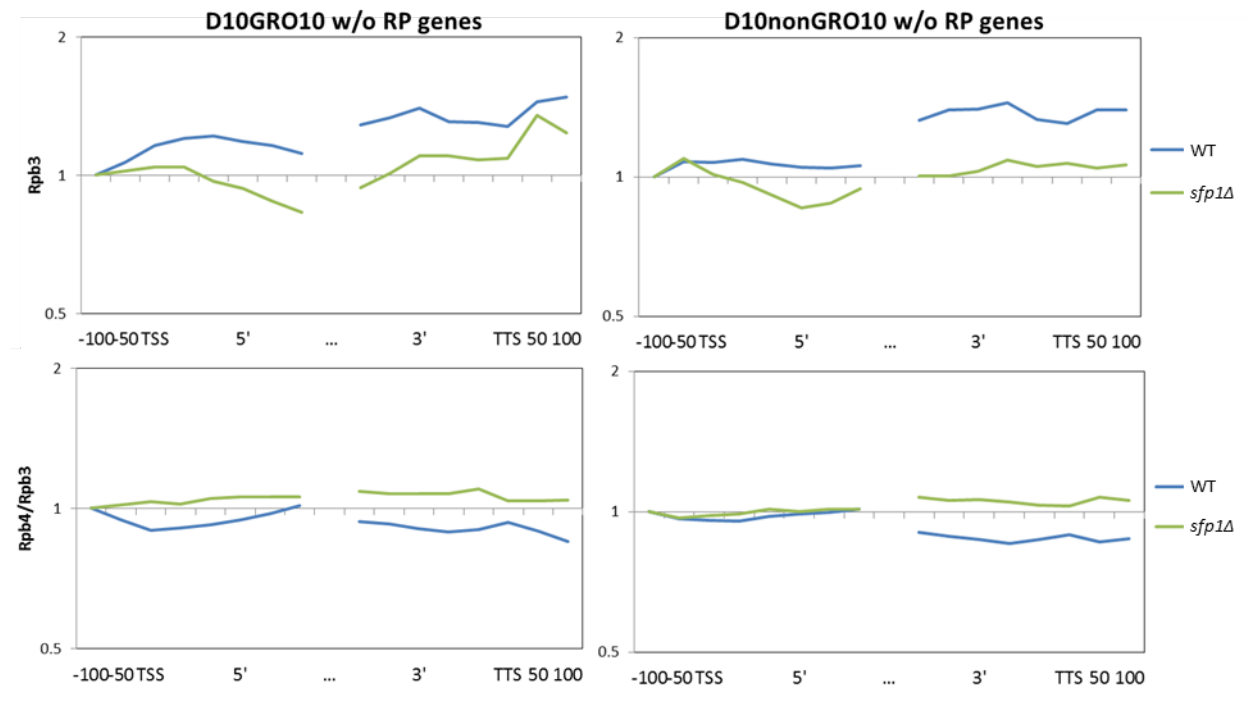
B
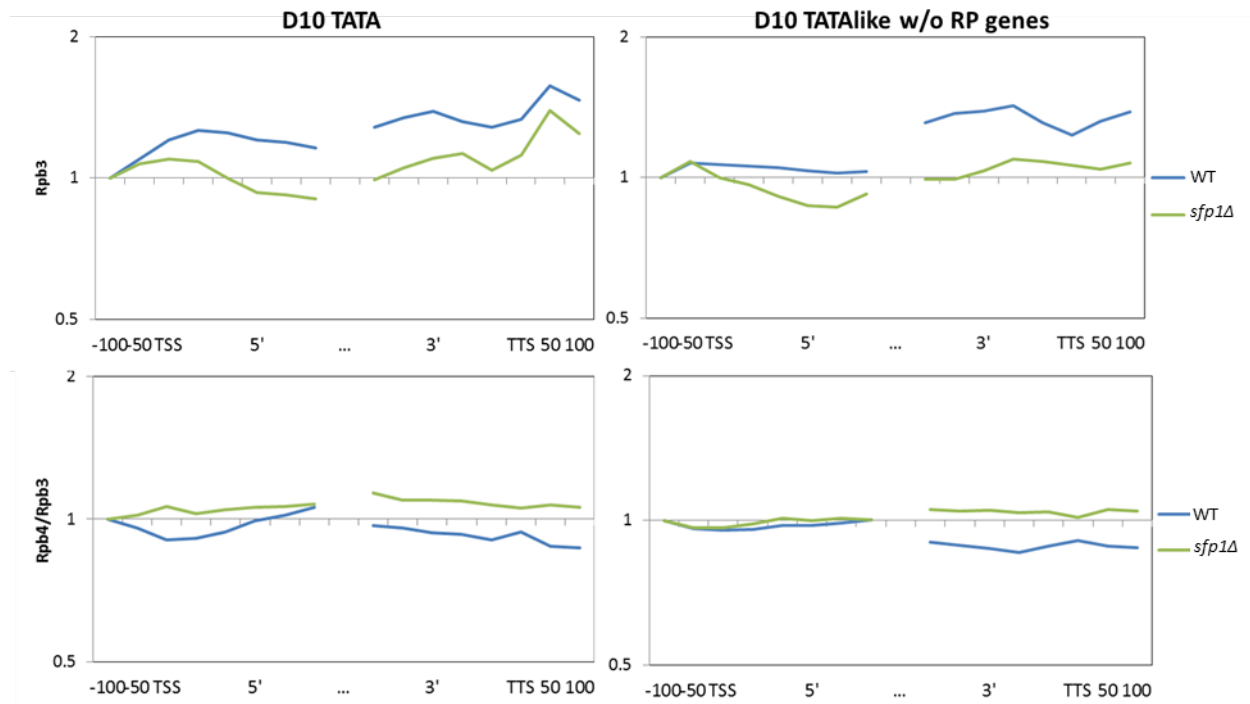
C
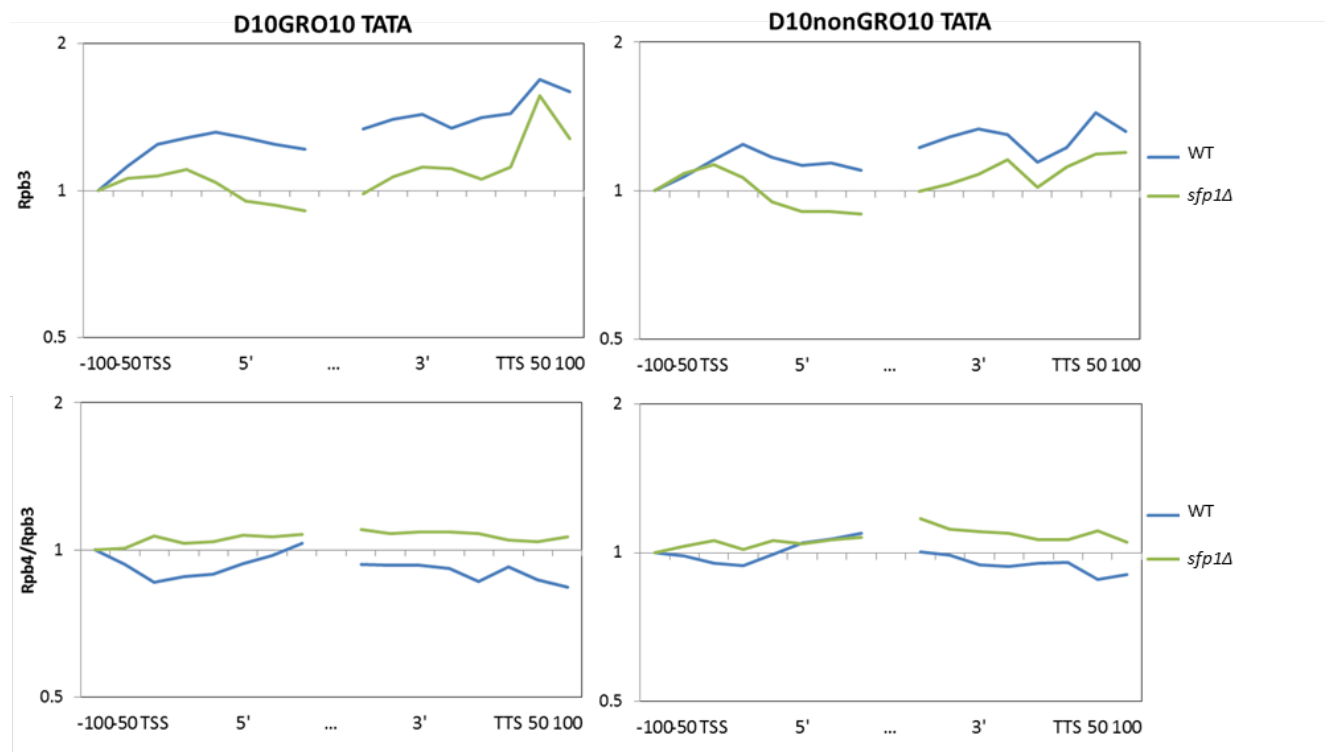
D
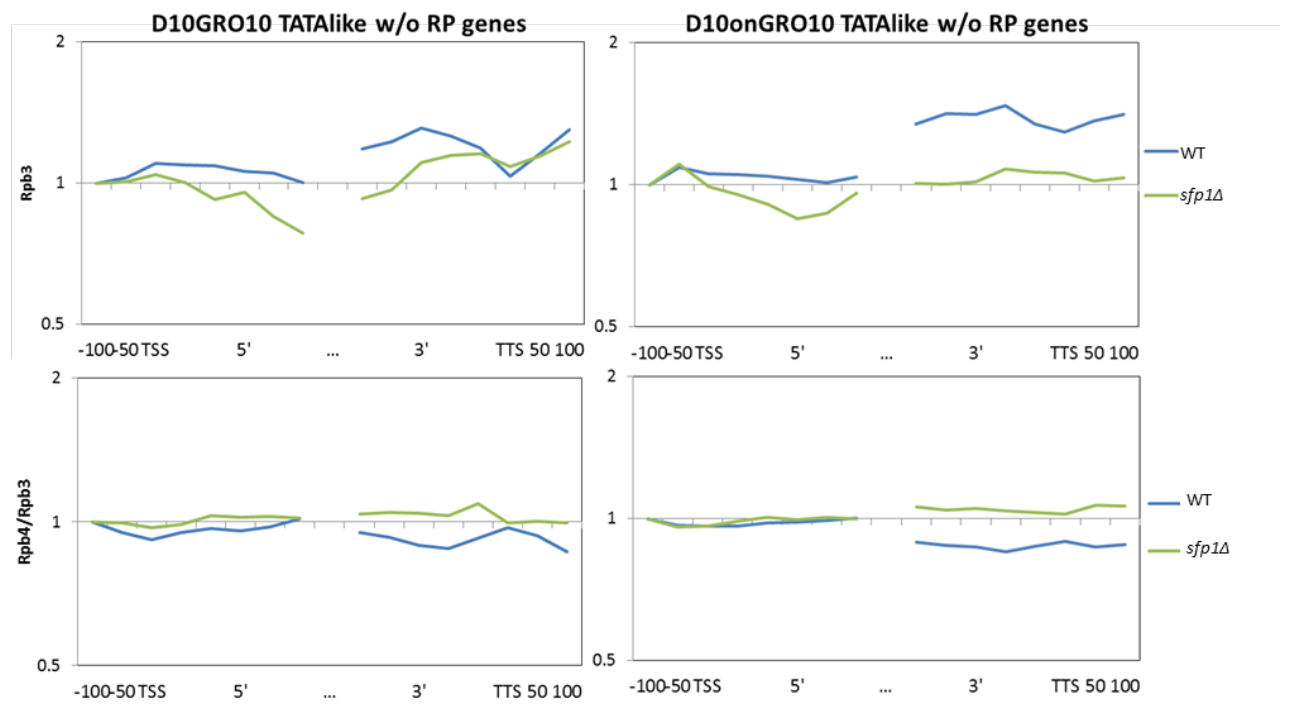
Figure 25 Rpb3 profiles and Rpb4/Rpb3 ratios in galactose in the following categories. RP genes have been excluded from all representations. **A**, D10 and GRO10, genes that present low backtracking and D10 non GRO10, genes more prone to backtracking. **B** Ratios in highly transcribed genes (D10 category), divided in TATA and TATA-like genes. **C** Ratios in the TATA genes divided in low (D10 and GRO10) and high (D10 non GRO10) backtrack categories. **D** Same as in (C) but for TATA-like genes. All the values were normalised to the -100 value in order to compensate the different levels of promoter-recruited polymerases in both strains.

Overall these results indicate that *sfp1Δ* causes a general effect in the dynamic of RNA pol II during transcription elongation and that this effect seems to be related to the conformation of the polymerase, as reflected by the Rpb4/Rpb3 ChIP ratios.

An unexpected result of our study is that TATA and TATA-like genes exhibit different RNA pol II profiles along the transcribed region, suggesting important differences in elongation and termination depending on the type of promoter core-element. Our results indicate that these differences between TATA and TATA-like genes provoke a different response to backtracking and to the RNA pol II alteration caused by *sfp1Δ.* The difference between TATA and TATA-like is, however, not intrinsic, since they attenuate in YPGal (compare figures 24 B and 25 B), explaining the more general impact of *sfp1Δ* in YPGal.

3.2.6. RNA polymerase II backtracking does not influence Ser2 phosphorylation by itself

We had previously studied the effect of *sfp1Δ* in Ser2P profiles along the genes (figure 14). Now we decided to observe the effect of *sfp1Δ* genome wide. In order to do so, we performed a ChIP on chip experiment against the Ser2 phosphorylated residue of the CTD and compared the levels with the total amount of Rpb3 along the genes, as we had previously done with Rpb4 and Rpb3. Since Ser2- phosphorylation is weak in the 5' end, we focused in the 3' end of the metagenes. We did not find significant differences in the Ser2-phosphorylated profiles after normalising for the level of Rpb3 (figure 26 A).

A
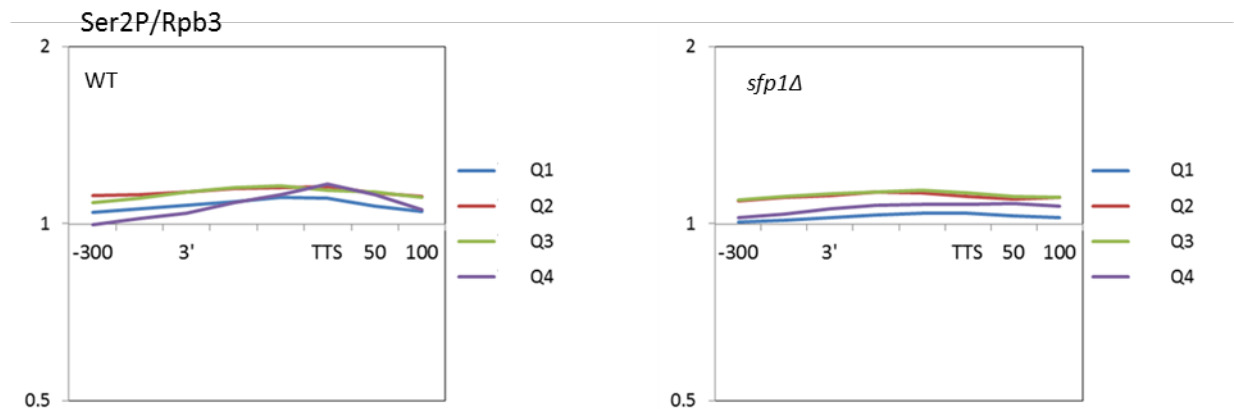
B
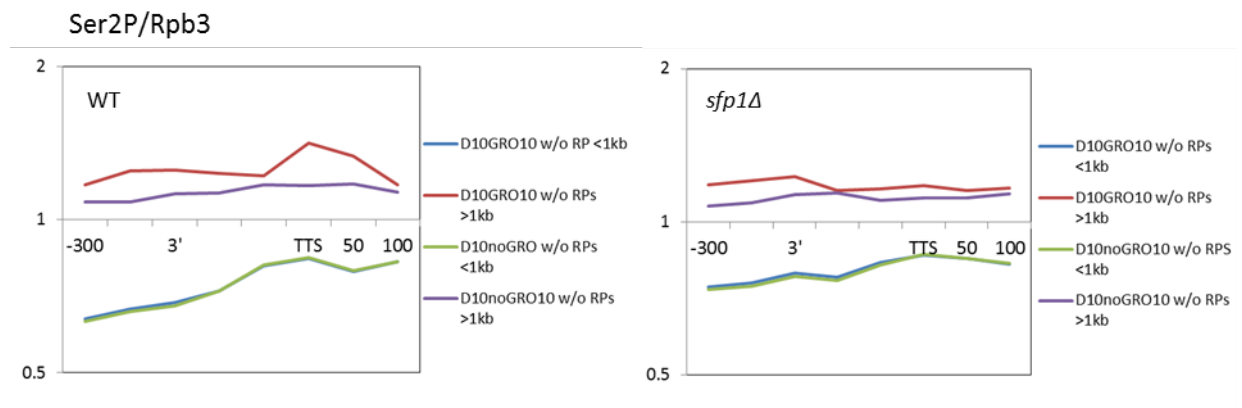
Figure 26 Ser2P/Rpb3 ratios in a wild type and a *sfp1Δ* mutant. **A** Ratios in the four groups of genes corresponding to four quartiles depending on the level of polymerases in a WT strain under normal conditions. **B** Ratios D10GRO10 and D10nonGRO10, in the WT and *sfp1Δ* strains. RP genes have been excluded from the list. Genes longer or shorter than 1 kb are shown separately.

We did not detect significant differences between TATA and TATA-like genes (not shown) or between D10GRO10 and D10nonGRO10, in either the wild type and *sfp1Δ* (figure 26 B). In contrast, we confirmed the effect of the gene length in Ser2 phosphorylation, as it has been previously described (Kim et al, 2010). This effect of gene length is not affected by *sfp1Δ* (figure 26 B). We conclude that RNA pol II backtracking has no direct effect on Ser2 phosphorylation.

3.2.6 Backtracking decreases RNA pol II elongation rate

The results shown so far indicate that *sfp1Δ* prevent RNA pol II backtracking in a wide set of highly transcribed genes. It would be expected that in a *sfp1Δ* background the polymerase showed an increased speed, considering that in the absence of Sfp1 the occurrence of backtracking is diminished. Experiments performed in our group by Dr Xenia Peñate indicate that in the absence of Sfp1 the RNA polymerase II presents an increased transcription rate (figure 27). These experiments were carried out by measuring the levels of RNA polymerases by ChIP (Rpb3 ChIP) and qPCR along the YLR454w gene body which was put under the control of the *GAL1* promoter. This gene is one of the longest genes in the *Saccharomyces cerevisiae* genome which makes it a useful tool to study RNA polymerase II progress during the transcription process (Mason and Struhl, 2005). The cultures were grown in galactose containing medium. After the addition of glucose the expression of *GAL1*::YLR454w is stopped and consequently there is no recruitment of new initiating polymerases to the promoter of the gene. However, the polymerases already engaged in the transcription of the gene will proceed until they reach the transcription termination site. Measuring the levels of those polymerases at different times allows us to determine their progression along the gene (see Materials and Methods). In order to be able to better compare these values the data for each probe was normalized by the value at time 0 (from the sample collected before glucose addition) and the average velocity was calculated. We observed how in *sfp1Δ* the polymerases appear to proceed at a higher speed than in the wild type (figure 27 A). The result shows an average velocity of 1.84 KB/minute for the wild type and 2.82 KB/minute for the *sfp1Δ* strain (figure 27 B). We conclude that backtracking significantly reduces the elongation rate of RNA pol II.

A

B
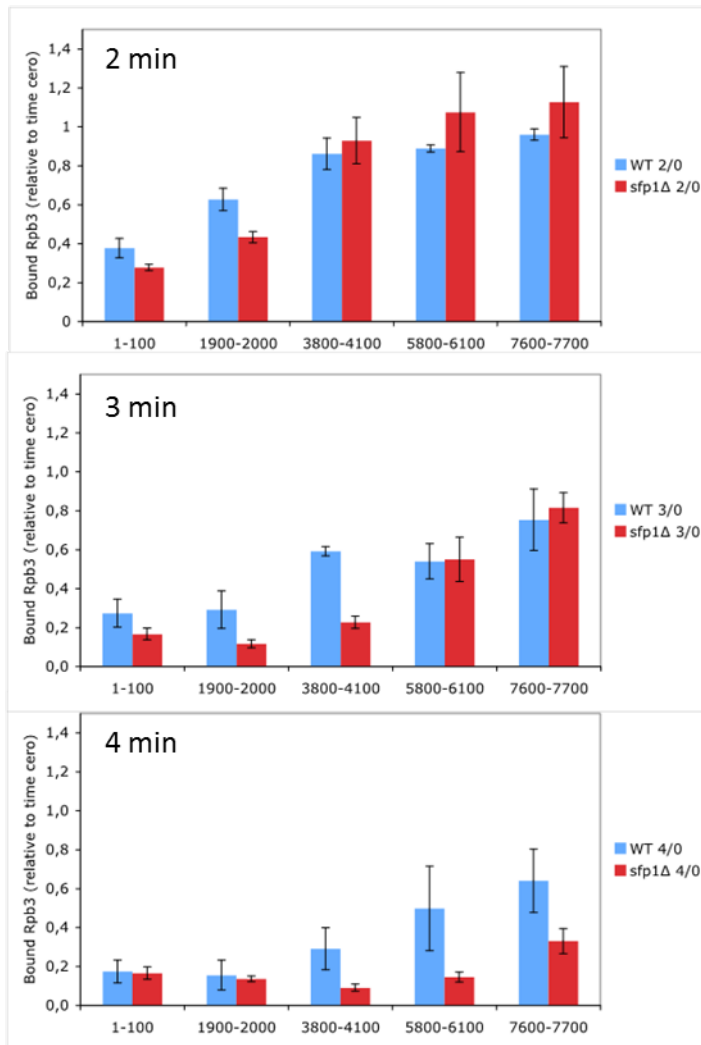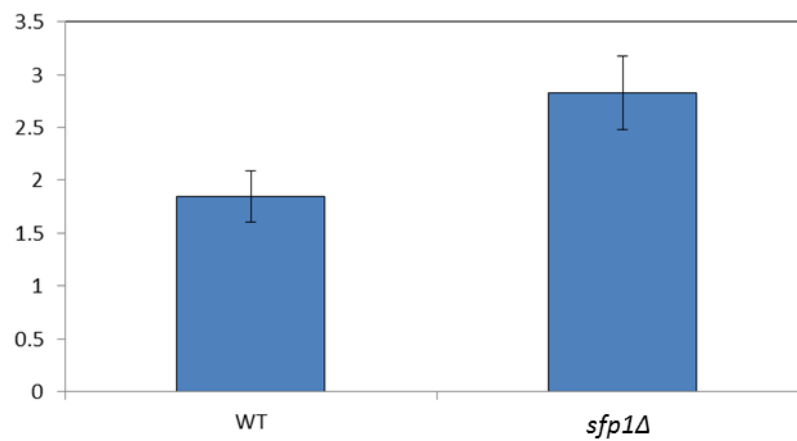
Figure 27 *sfp1Δ* increases RNA polymerase II elongation rate. **A** Variation in the levels of RNA polymerase II along the gene YLR454w at the indicated times upon the addition of glucose to galactose-grown cells, in wild type and *sfp1Δ* cells. All the values represent the average of three independent experiments and three different amplicons distributed along the gene. **B** Average rate of RNA polymerase II elongation speed in both WT and *sfp1Δ* strains. P value= 0.01 with an interval of confidence of 95%.

3.2.7   RNA pol II backtracking prevents drop-off

Next, we were interested in studying how the effect of Sfp1 affects RNA polymerase II activity. To do so we performed a run-on assay with the object of measuring the quantitative distribution of RNA polymerases along the gene YLR454w in the wild type and in *sfp1Δ*. Simultaneously, we performed ChIP experiments against Rpb3. In order to be able to compare both values, the probes used in the run-on membrane were equivalent to the amplicons designed to analyse the Rpb3 ChIP by qPCR. The activity of RNA polymerases in *sfp1Δ* appears to be higher at the 5'-end of the gene when compared to the wild type (figure 28 A). However, this activity decreases more steeply in the *sfp1Δ* than in the wild type being at the 3'-end of the gene approximately a half of that in the wild type. It should be noticed that although the activity of RNA polymerases is higher in the *sfp1Δ* strain, the level of total polymerases measured by anti Rpb3 ChIP is considerably lower than in the wild type (figure 28 B). The comparison of these two measurements indicates a higher specific activity in *sfp1Δ*. We quantified it by dividing active by total polymerases (figure 28 C). The specific activity of RNA polymerase II was higher in the *sfp1Δ* strain and particularly higher at the 5'-end of the gene (figure 28 C).

Anti Rpb3 data accounts for total polymerases including active and inactive (backtracked) molecules. The movement of RNA polymerases along the genes involve its active form. Therefore, run-on data are more informative than ChIP data in order to calculate RNA pol II drop-off rates. The drop-off rate was calculated considering the decrease in the run on signal from the first amplicon of the gene with the respect to the following amplicons. All the values were normalized to the first amplicon to allow a better comparison between both backgrounds, then for each experiment we calculated the slope of the tendency line of the representation of the values for each amplicon, which gave us an estimated value of the amount of active polymerases that initiated transcription with respect to those that reached the 3' end of the gene. We found that drop-off in the wild type was minimal during the first half of the gene (0-4 kbs), whereas it was significant in the 3' end (figure 28 D). In contrast, we

found a significant decrease of the run-on signal all along the gene in *sfp1Δ*. Considering the difference between the 5' and the 3' end that presented the wild type strain we also calculated separately the estimated drop-off on both ends. In figure 28 E we can see how the drop-off rate (proportion of RNA pol II molecules terminating per kb) in the wild type are significally lower than in a *sfp1Δ* at the 5' half (figure 28 E). The 3' the drop-off suddenly increases in the wild type compared to the *sfp1Δ* but this difference was also not significant. In the wild type the drop-off appears from the third amplicon onwards while the mutant strain shows a constant decrease in the activity of the polymerase from the 5' end of the gene. This result indicates that Sfp1 is preventing RNA polymerase II drop-off, especially at the 5' of the genes. Taking into account our previous results that show that Sfp1 induces RNA polymerase II backtracking we can hypothesize that Sfp1 is involved in a regulatory mechanism by which RNA polymerases fall into backtracking situations in order to undergo certain modifications that prevent this enzymes from dropping-off transcribing genes.

A

Run-on (active RNA pol II)

B

Rpb3 (total RNA pol II)

C

Run-on/Rpb3
(Specific activity of RNA pol II)

D

y = 5E-05x + 1.0353
R² = 0.6142

y = -0.0002x + 1.9709
R² = 0.8998

y = -0.0001x + 0.9551
R² = 0.8298

y = -0.0001x + 1.0092
R² = 0.8782
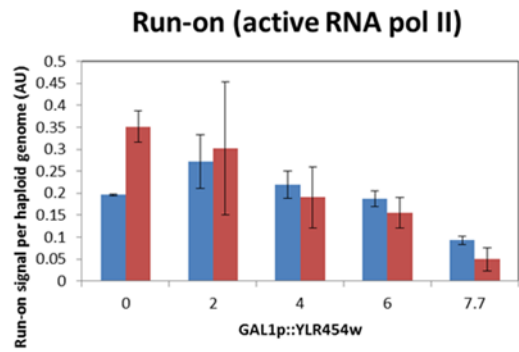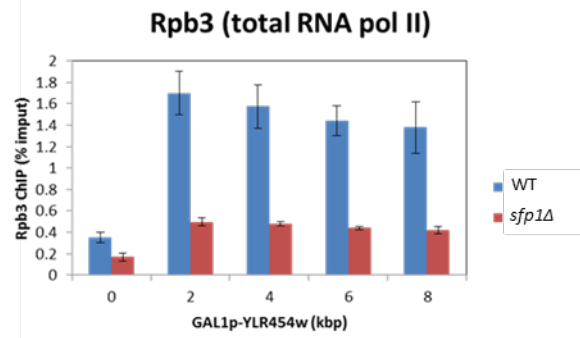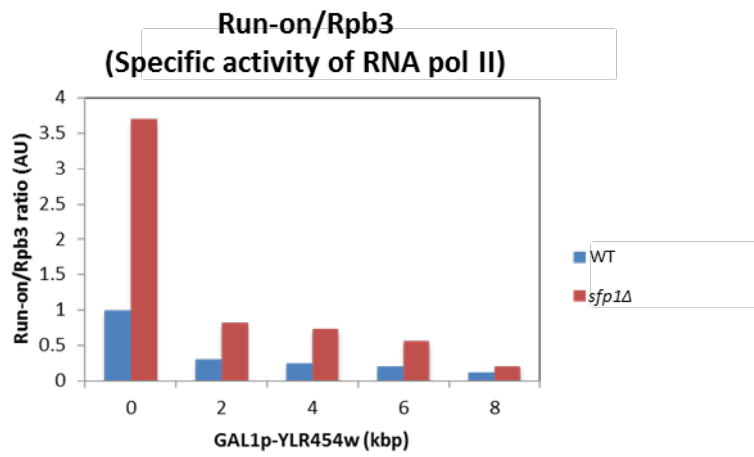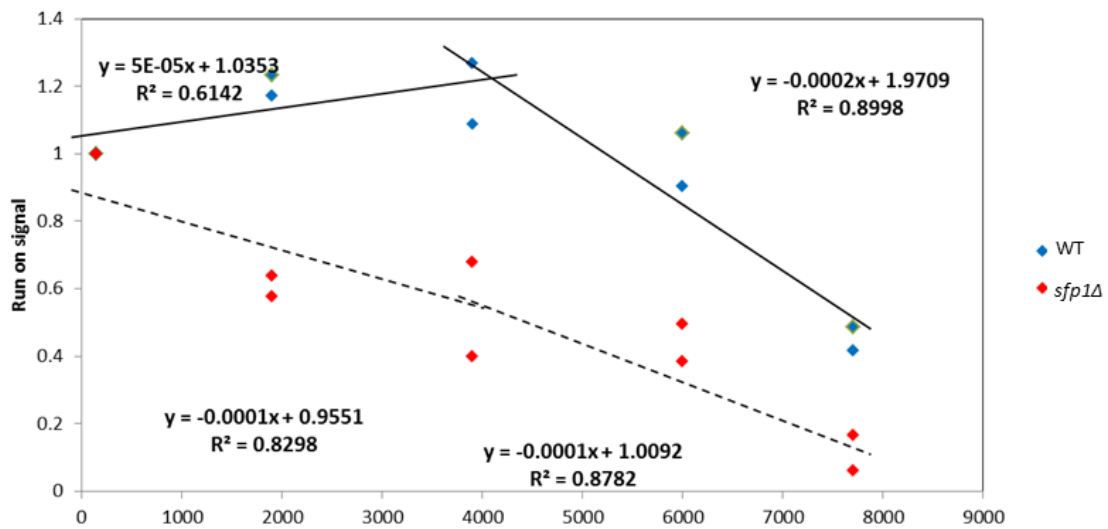
E
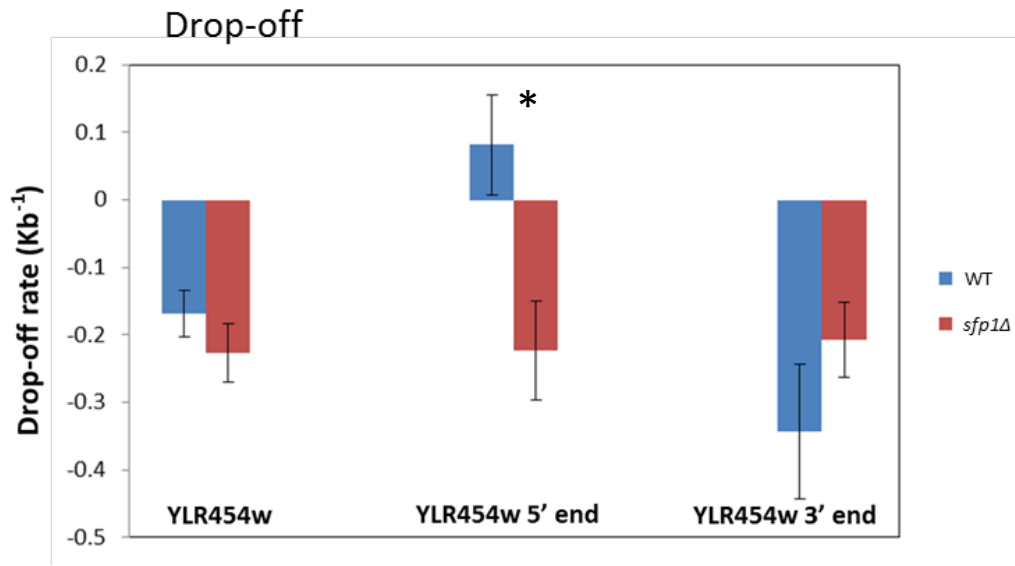
Figure 28 *sfp1Δ*-dependent suppression of RNA pol Ii backtracking increases drop-off. **A** Distribution of active polymerases as measured by run-on in a wild type and a *sfp1Δ* strains and **B** total polymerases measured by ChIP against Rpb3. All the experiments represent the values of three independent experiments. Error bars indicate standard error. **C** Specific activity of RNA polymerase II (run-on/Rpb3 ratio) along the gene YLR454w. **D** Representation of run on signal for all the experiments. **E** Drop-off rate calculated from the run-on data in both strains. The difference in the drop-off at the 5' end between the wild type and the *sfp1Δ* strains is statistically significant. The significance was determined by calculating the t value for each set of data.

4. DISCUSSION

4.1 RNA polymerase II backtracking in RP genes during nucleolar stress

A change in RP genes' expression allows RNA polymerase II dependent transcription to support cell growth in the absence of TFIIS under severe NTP stress. We propose that TFIIS is required particularly for the transcription of RP genes under NTP-depletion conditions. The detailed transcriptional analysis of some RP genes supports this view. We found that, upon 6AU treatment, the TFIIS/RNA polymerase II ratios of RP genes are substantially higher than those exhibited by non-ribosomal genes, including strongly transcribed ADH1 (figure 2 C). As a structural work reveals, the TFIIS–RNA pol II complex is incompatible with the RNA polymerization reaction (Cheung et al, 2011). Accordingly, TFIIS' recruitment likely responds to a previous backtracked configuration, and should involve the reactivation of arrested RNA polymerases. In fact, the preferential occupancy of RP genes by TFIIS in response to 6AU (figure 2 C) agrees with the strongly increased RNA pol II specific-activity found in RP genes, as reflected by the run-on/Rpb3 ratios (figure 4 C). The simplest explanation for this correlation is that TFIIS is preferentially recruited to those elongating RNA polymerase II molecules which become backtracked in RP genes when NTPs are scarce. This hypothesis involves a greater tendency of RNA polymerase II to become backtracked in RP genes than in non-RP genes.

This is, in fact, one of the conclusions drawn by a previous study from our lab, which revealed that RP genes exhibit the highest RNA pol II ChIP/run-on ratios throughout the genome, interpreted as the highest proportion of arrested RNA pol II (Pelechano et al, 2009). Similarly, a nascent elongating transcript sequencing (NET-seq) database has revealed a high frequency of pausing in RP genes (Churchman and Weissman, 2011). Alternatively, the relatively high TFIIS/Rpb3 ratios exhibited by RP genes upon 6AU treatment could well be the result of a longer resident time of the TFIIS–RNA polymerase II complexes under down-regulation conditions. Reactivated RNA polymerase II (after RNA cleavage) has been described to stay in place for a time before resuming transcription (Churchman and Weissman, 2011). One possible cause for this delay may be the slow kinetics of TFIIS dissociation. In a down-regulation situation of RP genes, provoked by NTP stress, this slow TFIIS off-rate could involve a transient enrichment in the TFIIS–RNA polymerase II complexes, these being competent complexes for run-on. In either of the alternative explanations we offer herein, the higher TFIIS/Rpb3 ratios exhibited by RP genes and the differential effect of *dst1Δ* indicate a crucial role for TFIIS in the transcription of RP genes. The aforementioned work also demonstrated that the RNA polymerase II ChIP/run-on ratio of RP genes could be regulated. Mutants like *tpk2Δ*, affecting RP genes' response to the Ras–PKA pathway, or *RAP1ΔSIL*, affecting the main gene-specific transcription factor of RP genes, caused a substantial increase in the proportion

of active RNA pol II (run-on signal) without changing the relative amount of bound polymerase (ChIP signal) (Pelechano et al, 2009). We thought it most significant that the same mutations could suppress the sensitivity of *dst1Δ* to 6AU (Gomez-Herreros et al, 2012).

In a different study, we also described how RP genes display the lowest 3'/5' ratios of active transcription (measured by run-on) of the yeast genome (Rodríguez-Gil et al, 2010). All these data indicate that the likelihood of RNA polymerases II molecules pausing and backtracking is greater in RP genes than in most other genes and, therefore, the requirement of TFIIS is maximal in RP genes. We assume that all the RNA polymerase II molecules are able to produce similar run-on signals, at least on average. We base this assumption on the stringent run-on assay conditions, which should provide a homogenous template (nucleosome-free DNA) for each elongating RNA polymerase (Hirayoshi and Lis, 1999). Alternative interpretations of the run-on signal variation, i.e. a different length of the run-on transcript, would change the molecular meaning of our results, but would not change the main message of this work: the differential transcriptional behaviour of the RNA polymerase II molecules when transcribing RP genes and their higher dependency on TFIIS in comparison with other highly transcribed genes.

It has been demonstrated that backtracking and RNA cleavage are very common phenomena and that they are likely consubstantial to RNA polymerase II-dependent transcription in vivo (Mason and Struhl, 2005; Churchman and Weissman, 2011). This is in good agreement with previous in vitro experiments (Galburt et al, 2007). However, life without TFIIS is possible in yeast under standard growing conditions since *dst1Δ* mutants are viable. This viability is explained by the basal intrinsic cleavage activity of RNA polymerase II, which takes place even in the absence of the stimulatory influence of TFIIS (Weilbaecher et al, 2003; Sigurdsson et al, 2010). Yet under NTP-depleting conditions, the frequency of backtracking in RP genes in the absence of TFIIS would be so high that cells would undergo a RP shortage and would eventually become unviable. RNA polymerase I ChIP data do not suggest that this imbalance is caused by the up-regulation of rDNA transcription (figure 1B) (Gómez-Herreros et al, 2012). These results favour the conclusion that TFIIS contributes to the coordination of the genome fraction which encodes ribosomal elements. We conclude that RP genes are the main targets of TFIIS in the response of ribosome biogenesis to 6AU.

Upon a treatment with NTP depleting drugs happens an imbalance between RP genes expression and RNA polymerase I transcription, that can be overcome by the presence of TFIIS (Gómez-Herreros et al, 2012; Gómez-Herreros et al, 2013). RNA polymerase I dependent transcription takes place in the nucleolus, the nuclear subdomain formed by the genes encoding rRNAs and where these rRNAs are assembled into ribosomal subunits. The high number of rDNA tandem repeats are located in Chromosome XII.

Our experiments show that RP genes relocate to positions closer to the nucleolus upon 6AU treatment. Many studies have described the movement of genes towards the nuclear pore complex to facilitate the export of the mRNAs, a mechanism denominated gene gating (Burns and Wente, 2014) but it is the first time genes are described to locate closer to the nucleolus under certain conditions.This could be one of the mechanism by which the cell responses to nucleolar stress. This mechanism would not affect all the genes in general, but only those genes like RP genes that need a coordinate transcription with rDNA. The movement of RP genes towards the nucleolus and the higher backtracking frequency of these genes upon 6AU treatment would be part of a particular regulatory mechanism particular of RP genes in response to nucleolar stress.

The transcription rate of these rDNA units in humans has been described to be dependent on UBF, a HMG protein (Stefanovsky et al, 2001). In yeast, the factor Hmo1 shares some similarities with UBF and it is necessary for rDNA transcription (Gadal et al, 2002; Hall et al, 2006). Interestingly this protein also plays a positive role in RP genes transcription acting on the promoters of these genes, where it physically interacts with Rap1. It has been hypothesised that Hmo1 coordinates RP genes and rRNA transcription (Berger et al, 2007; Hall et al, 2006). It has also been proposed that the coordination between RP genes and rRNA transcription could be coupled by the CURI complex which is a complex formed by the RP regulator Ifh1, and the pre-rRNA processing factors Utp22 and Rrp7 (Rudra et al, 2007). It has yet to be determined if both mechanisms could be related.

Our results indicate that RP genes localise closer to the nucleolus under transcriptional stress conditions. The fact that TFIIS is necessary for this to happen could mean that TFIIS actively contribute to this gene movement or that only those genes that are being actively transcribed undergo this process. The highest proximity of RP genes to the nucleolus occurs after 90-120 minutes of treatment with 6AU (figure 6). This happens after the recovery of the RP/ rDNA transcriptional balance (Gómez-Herreros et al, 2013).

The recruitment of TFIIS to RP genes in response to 6AU can be observed as soon as 15 minutes after the addition of the drug (figure 2 C). The activity of the RNA polymerase II (ratio run-on/Rpb3, is recovered at 90 minutes of 6AU treatment in RP genes (figure 4 C). This supports an indirect effect of TFIIS in RP genes relocation to the nucleolus. In this scenario, TFIIS recruitment to RP genes would be an early response to nucleolar stress and gene relocation a latter component of this response (figure 29).
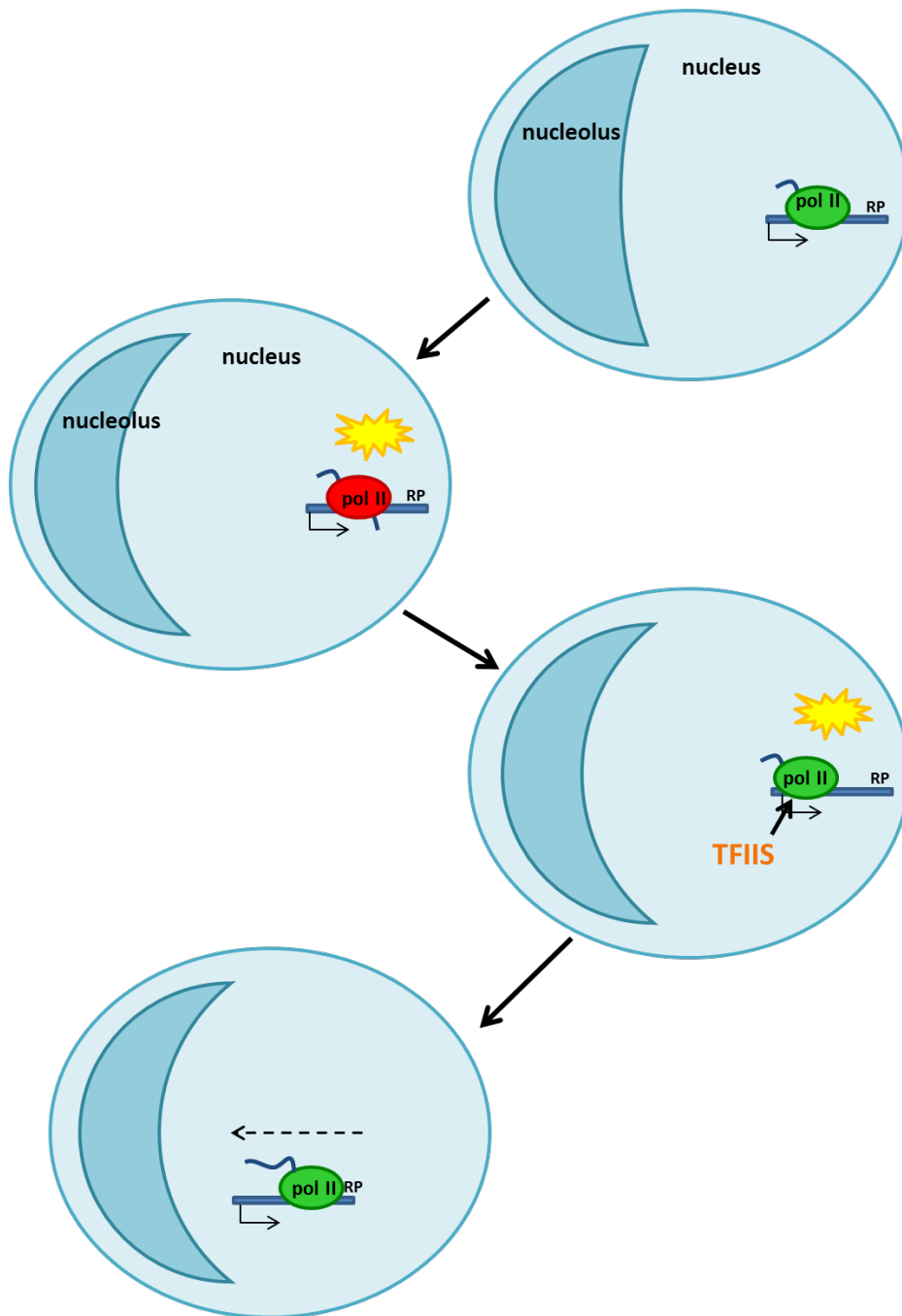
Figure 29 Model explaining RP genes relocation to the nucleolus as a late response to nuclear stress.

4.2 RNA polymerase II backtracking across the genome

The sensitivity to NTP-depleting drugs caused by the absence of TFIIS can be suppressed by the mutation of some of the genes affecting the regulation of ribosome biogenesis (Gómez-Herreros et al, 2012), like *SFP1*. Sfp1 controls the transcription of ribosomal related genes in response to nutrients (Jorgensen et al, 2002). One of the roles of Sfp1 and other RP genes regulators is the communication of the growth potential to ribosome synthesis (Jorgensen et al, 2004). This fact suggests that the *sfp1Δ* mutation causes major alterations in the regulatory mechanisms of ribosomal genes to the extent that the arrest-prone scenario of RP genes would be prevented, thus alleviating their dependency on TFIIS under NTP stress. When Sfp1 is absent, RNA pol II molecules transcribing RP genes are not significantly affected by 6AU in either a *dst1Δ* or a wild-type background (figure 10), in spite of the clear impact of 6AU on rDNA transcription (figure 11).

The result showed in figure 13 indicates that, in the absence of Sfp1, TFIIS is not only dispensable to resist NTP-depleting drugs but that this factor is not recruited to RNA polymerase II transcribed genes. This could mean that when Sfp1 is absent, the polymerases do not need the function of TFIIS. One possible explanation of this phenomenon would be that in these conditions the RNA pol II backtracking is not taking place. In accordance to this, our results let us conclude that *sfp1Δ* is provoking an effect on RNA polymerase II-dependent transcription and that this effect is not limited to RP genes. These results indicate a possible effect of Sfp1 upstream of the function exerted by TFIIS. Apparently, Sfp1 would have an antagonistic effect on RNA polymerase II dependent transcription elongation by inducing the backtracking of the polymerases. This effect, contrary to what has been previously described about the factor Sfp1 (Jorgensen et al, 2004) would not only affect RP genes or ribosomal biogenesis genes but it would be a general effect for RNA polymerase II dependent genes as we can see in figure 10 where not only the polymerases that are transcribing RP genes change their behaviour in a *sfp1Δ* background when compared to the wild type. Actually, when we observe the active versus total polymerases ratio (figure 10 C) we can see that all the genes studied were insensitive to a 6AU treatment whereas in a wild type or a *dst1Δ* mutant NTP depleting drugs have a general effect (figure 10).

We consider very unlikely a direct general effect of Sfp1 on RNA pol II backtracking. We rather consider that the regulatory consequences of *sfp1Δ* on growth genes indirectly cause this phenomenon. In fact, other mutations affecting RP regulation can suppress the sensitivity of *dst1Δ* to 6AU as well (Gómez-Herreros et al, 2012). Either direct or indirect, the effect of

sfp1Δ on RNA pol II backtracking makes this mutation a very useful tool to investigate this phenomenon genome-wide.

As shown in figure 14, the *dst1Δ* mutant shows a defect in the phosphorylation of Ser2 as can be seen when the ratios Ser2P versus Rpb3 are compared between a wild type and a *dst1Δ* strains. This defect in Ser2P in the mutant could be caused by the increased time that the polymerases remain backtracked when TFIIS is not present. This defect is suppressed by the absence of Sfp1 (figure 14 B) in a similar manner it rescued sensitivity to 6AU in a *dst1Δ* background. This indicates that the decrease in Ser2P levels is due to unsolved backtracking events. However, when we look at the genomic data we can observe that there is no significant difference in Ser2P between the wild type and *sfp1Δ* (figure 26) strains indicating that backtracking itself is not affecting the levels of Ser2P in a general manner.

The general effect of *sfp1Δ* led us to use this mutation as a tool to study RNA pol II backtracking on different groups of genes. Thanks to the genomic data obtained from ChIP on chip and GRO experiments we were able to represent the level of total (Rpb3) and active (GRO) polymerases across the yeast genome in the wild type and the *sfp1Δ* strains. When we compared the two sets of data we found a much narrower range for Rpb3 ChIP than for GRO in *sfp1Δ* (figure 16). This is fully compatible with a general effect of *sfp1Δ* in RNA pol II backtracking and is clearly exemplified by RP genes (figure 16). The definition of Rpb3/GRO ratios allows us to quantify the tendency of RNA polymerases when transcribing a given gene. These ratios are higher in highly transcribed gene than in those genes with low levels of transcribing polymerases (figure 17 A). Z-scores allowed us to make a comparison between wild type and *sfp1Δ* ratios. Although this approach does not tell us information on absolute levels of backtracking in *sfp1Δ* and in the wild type, it shows that highly transcribed genes in *sfp1Δ* have a lower tendency of backtracking, compared to the genome average, than the same set of genes in the wild type (figure 17 A). The genes that present higher probability of backtracking, those that belong to the D10nonGRO10 group, are the genes that show the most significant difference between the wild type and *sfp1Δ* strains when we analysed the Rpb3/GRO ratios. This result supports our hypothesis that *sfp1Δ* is causing some kind of effect that makes the polymerases less prone to backtracking. It could be argued that the higher ratio of D10GRO10 versus D10nonGRO10 was due to higher hypothetical levels of Rpb3 of this last group. However the levels of Rpb3 are very similar, in both groups (figure 17 B). We concluded that transcription elongation dynamics is different in these two groups of genes, making RNA pol II backtracking differentially frequent. This differential elongation dynamics is reflected in

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the Rpb3 profiles (figure 24). In parallel to its effect on backtracking, *sfp1Δ* impacted dramatically the Rpb3 profiles of D10nonGRO10 genes, whereas it had only minor impact on D10GRO10 genes (figure 24 A). The differential effect was more evident in the 5' end.

The genes of *Saccharomyces cerevisiae* can be divided in two groups depending on the nature of their promoters, TATA and TATA-like (previously denominated TATA-less) genes (Rhee and Pugh, 2012). TATA genes are associated with responses to stress, and they are tightly regulated, possibly to reflect the need to balance stress response with housekeeping functions (Basehoar et al, 2004). Chromatin might play a role in the promoter of TATA genes by keeping the TATA-box sequences inaccessible until it is make available to the transcription machinery by the intervention of activators (Struhl, 1999). TATA and TATA-like promoters have different regulatory mechanisms (Basehoar et al, 2004). TATA genes are highly regulated by nucleosomes, chromatin regulators and TBP regulators compared to TATA-like genes (Basehoar et al, 2004). In the transcription of TATA genes intervene the SAGA complex while in TATA-like there is a predominant role of TFIID (Huisinga and Pugh, 2004; Basehoar et al, 2004) although TFIID can also function at SAGA regulated genes when it is absent (Lee et al, 2000). The D10GRO10 category of genes is more enriched in TATA genes than TATA-like genes, and the D10nonGRO10 is more enriched in TATA-like (figure 18 B). These genes (D10nonGRO10 TATA-like) are more sensitive to *sfp1Δ*: lower levels of total RNA pol II without significant variation in run-on (active RNA pol II) (figure 19).

We detected a different dynamic of RNA polymerase II elongation in those two groups of genes (TATA and TATA-like) as can be seen in figure 24 B. Interestingly, the differential elongation dynamics between D10GRO10 and D10nonGRO10 in the wild type was only observed in TATA-like genes (figure 24 C, D). Accordingly, only in TATA-like genes *sfp1Δ* produced a differential impact between D10GRO10 and D10nonGRO10 (figure 24 C, D). This means that the mechanisms producing RNA pol II backtracking in TATA and TATA-like genes should not be the same. In the case of TATA-like genes it is sensitive to *sfp1Δ,* whereas in TATA genes it is not. In galactose, where both TATA and TATA-like genes were responsive to *sfp1Δ* (figure 21), TATA genes show closer Rpb3 profiles to TATA-like genes than in glucose (compare figure 24 B and 25 B). We envisage two possibilities that could make TATA and TATA-like genes behave differently with respect to transcription elongation and backtracking. One possibility is that the elongation machinery of RNA pol II and/or the configuration of the enzyme itself could be different between these two groups of genes. Rpb4/Rpb3 ratios along the transcription units support this hypothesis.

In TATA genes Rpb4/Rpb3 ratios decrease during transcription initiation and recover up in the polyA region, falling down again during termination (figure 24 B). We interpret this variation of Rpb4/Rpb3 down and up during transcription as a consequence of changes in the configuration of the elongation complex (RNA pol II plus elongation factors) rather than as a real change in the subunit composition of RNA pol II during elongation. Depending on the configuration of the elongation complex, the relative capability of Rpb4 and Rpb3 to cross-link DNA would change. Rpb4 forms a heterodimer dissociable from the catalytic core of the RNA polymerase II together with Rpb7. It has been proposed that Rpb4 is involved in the communication between the nucleus and the cytoplasmic machineries that determine the levels of mRNAs in the cell (Lotan et al, 2005). Rpb4/7 binds to nascent transcripts (Ujvari et al, 2006) and it is exported from the nucleus to the cytoplasm where it would play a post-transcriptional role (Selitrennik et al, 2006). It has been described to have a role in mRNA degradation through its association with mRNA decay factors and P-bodies (Lotan et al, 2005; Lotan et al, 2007). In general, the Rpb4/7 heterodimer would associate with the mRNA throughout its life cycle, acting as an mRNA coordinator (Halel-Sharit et al, 2010). The final decrease of Rpb4/Rpb3 ratios in the termination region of TATA genes might be reflecting the transfer of Rpb4/7 to the nascent mRNA molecule.

In TATA-like genes, Rpb4/Rpb3 ratios also decrease during initiation, but they do it less intensively in the 5' end and more deeply in the 3' end (figure 24 B). Moreover, there is no significant increase of Rpb4/Rpb3 ratios in the polyA region (figure 24 B). This profile is compatible with a different dynamic of the Rpb4/7 heterodimer during elongation and its putative transfer to nascent mRNAs. We conclude that Rpb4/Rpb3 ratios support a different dynamic in the configuration of the RNA pol II elongation complex in TATA and TATA-like genes. This differential dynamic of the elongation complex is suppressed by *sfp1Δ*. In this mutant all groups of genes analysed show similar Rpb4/Rpb3 profiles along the transcription unit, without any decrease during initiation but rather a slight increase (figure 23, 24 and 25). However, *sfp1Δ* only suppresses backtracking in D10nonGRO10 TATA-like genes; so, backtracking in TATA genes is not influenced by the drastic changes in RNA pol II configuration caused by *sfp1Δ*. It is necessary a second element that helps explaining the backtracking and this could be the chromatin structure of the genes. Differences in chromatin dynamics can be the cause of the different behaviour of these groups of genes. When the polymerase proceeds through nucleosomes during transcription elongation, the nucleosomes are remodelled to allow the passing of the enzyme. Upon remodelling two alternative mechanisms can occur (reviewed in Gómez- Herreros et al, 2012-2). The histones can be evicted by a specific set of factors in a process called histone eviction, or they can be rearranged in the hexasome, that is

a modified nucleosomal core where only a H2A/H2B dimer has been evicted (Kireeva et al, 2005). In this last mechanism it's expected that the polymerases would show a greater tendency to backtrack when they go through the remodelled nucleosome. Transcription of TATA genes has been correlated to histone eviction (Schwabish and Struhl, 2004). In our laboratory it has been found a strong functional conection of prefoldin and histone eviction, preferentially operating in TATA genes (Millán-Zambrano et al, 2013). Moreover, prefoldin mutants exhibit synthetic interactions with *dst1Δ* in growth and in gene expression suggesting that prefoldin and TFIIS work in different pathways.

Altogether, we propose a model where TATA genes would usually be transcribed by RNA pol II following a histone eviction dynamic, whereas TATA-like genes would be transcribed following a hexasome mode of chromatin dynamics (figure 30). These alternative modes of elongation would involve different set of factors imposing alternative configurations to the elongation complexes. The alteration produced by *sfp1Δ* would change these configurations provoking a modification in the elongation dynamic of TATA-like genes that would make it less prone to backtracking in their chromatin context.
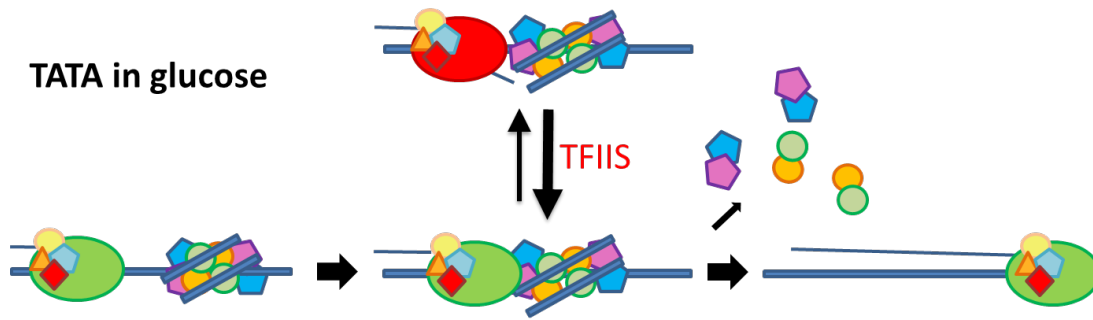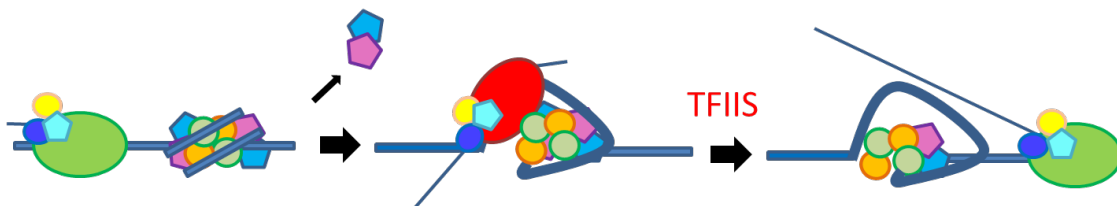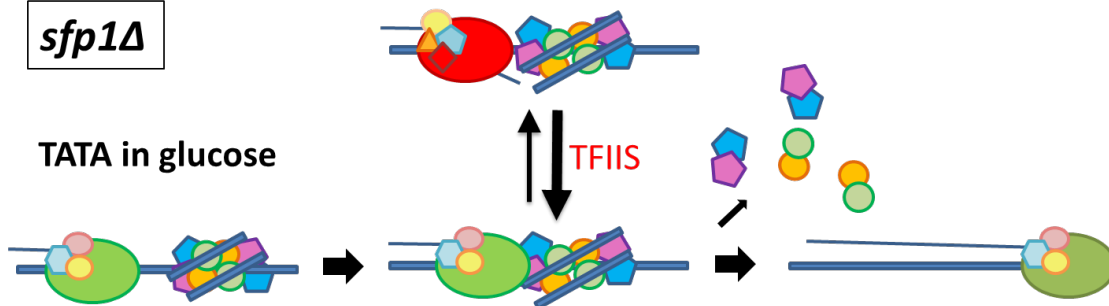
Figure 30 A model explaining the relationship between RNA pol II backtracking, chromatin dynamics and elongation processivity. Green ovals represent active RNA polymerase II, red ovals, backtracked RNA polymerase II. TATA genes chromatin dynamics correspond mostly to the histone eviction mode while TATA-like genes and also TATA genes in galactose would follow the hexasome mode of chromatin dynamics. Under the hexasome mode, RNA pol II backtracking would prevent drop-off.

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The exception for TATA genes would be the termination region where they show significant higher levels of RNA pol II in *sfp1Δ* compared to the wild type (figure 24 B, C). We have proposed that termination by RNA pol II involves backtracking (Jordan-Pla et al, 2015) and is linked to the action of the exosome on the 3' end of backtracked mRNA (Lemay et al, 2014). The decrease in backtracking tendency produced by the effect of *sfp1Δ* in the configuration of RNA pol II would produce a delay in termination and a consequent increase of RNA pol II levels in the termination region of TATA genes.

4.3 Backtracking decreases RNA polymerase II speed and favours its processivity

We have demonstrated that RNA pol II backtracking is less frequent in *sfp1Δ* than in the wild type. Consequently we could expect those polymerases that are not been detained during transcription elongation to proceed faster along the transcribed genes. The results obtained in our lab confirm this hypothesis (figure 27). If the polymerase proceeds faster in a *sfp1Δ* strain it could be expected a higher mRNA production in this background. But this is not the case, in *sfp1Δ* a decrease in the concentration of mRNA can be observed (Daniel Medina, PhD thesis university of Valencia). This contradiction can be explained in two ways. Either *sfp1Δ* provokes a decrease in the stability of mRNA or it reduces the procesivity of RNA pol II.

We did not measure mRNA stability in *sfp1Δ* but we found a clear decrease in the RNA pol II processivity in *sfp1Δ* compared to the wild type in the long YLR454w transcription unit under the *GAL1* promoter. According to our results, the reduced mRNA levels of *sfp1Δ* would be explained, at least partially, by the higher drop-off rate of RNA polymerase II in this background (figure 28). Drop-off was especially intense in the 5' half of the YLR454w gene (figure 28 D, E). We also found a stronger impact of *sfp1Δ* in the RNA pol II dynamics on the 5' end of TATA-like genes in glucose (figure 24 B) and on both TATA and TATA-like genes in galactose (figure 25 B). So we conclude that backtracking contributes to RNA pol II processivity and that this phenomenon is particularly important in the 5' end of the genes.

The drop-off rate of RNA pol II in a wild type background has been calculated to be on average a 20% per kilobase in *S. cerevisiae* (Sun et al, 2012). We found a similar value in our experiments with YLR454w (figure 28). The gene YLR454w is significantly longer (more than 8 kb) than the average of genes in *S. cerevisiae.* In the wild type we only detected drop-off in the 3' end of this gene. Since most of the genes of *Saccharomyces cerevisiae* are shorter than 2 kb, we consider that the actual impact of *sfp1Δ* in RNA pol II drop-off across the yeast genes would be stronger than our measurement in the long model gene.

Taking into account all our results, we propose that RNA polymerase II backtracking prevents the enzyme from dropping-off the genes that are being transcribed in a hexasome-dependent maner (figure 30). In glucose, this contribution of backtracking to gene transcription would be more relevant in certain groups of genes (TATA-like genes, which include RP genes). This way, it could be hypothesised that genes with house-keeping functions could use backtracking as a strategy for increased processivity that would allow these genes to maintain a permanent chromatin at the cost of a lower velocity while genes of regulated transient expression could afford a full histone eviction dynamic, allowing a quick response.

5. CONCLUSIONS

1. TFIIS is recruited to RP genes during nucleolar stress caused by NTP depletion, in response to the accumulation of backtracked RNA pol II on these genes.

2. In a later step during nucleolar stress reactivated RP genes relocate closer to the nucleolus.

3. RNA pol II backtracking is a frequent phenomenon across the yeast genome and its incidence in highly transcribed genes depends on their promoter architecture and on the configuration of the enzyme.

4. Highly transcribed TATA and TATA-like genes undergo different transcription elongation dynamics.

5. RNA polymerase II backtracking decreases the elongation rate of the enzyme but contributes to its processivity.

6.

6. MATERIALS AND METHODS

6.1 Culture media and growth conditions

6.1.1 Yeast culture media.

-YPD rich medium: 1% yeast extract; 2% bacto-peptone; 2% glucose.

- YPD+G418 Yeast rich medium: YPD medium supplemented with G418 sulphate to a final concentration of 200ml/l after autoclaving.

- YPGAL rich medium: 1% yeast extract; 2% bacto-peptone; 2% galactose.

- SD minimal medium: 0.17% yeast nitrogen bases (YNB) without aminoacids and ammonium sulphate; 0.5% ammonium sulphate; 2% glucose.

- SC complete medium: SD medium supplemented with the aminoacids leucine, tryptophan, histidine, lysine and nitrogenous bases adenine and uracil. In case one or more of these requirements had been omitted from the medium it would be specified.

-Sporulation medium SPO: 1%potasium acetate; 0.1% yeast extract; 0.005% glucose. It was supplemented with the same requirements described for SC at one fourth of their usual concentration.

Solid media were prepared by the addition of 2% agar before autoclaving.

6.1.2 Growth conditions

Yeast strains were cultured at 30°C, except in the cases where it is specified otherwise. Liquid cultures were grown in orbital shaking incubators.

6.2 Antibiotics, drugs, enzymes and inhibitors

-Bovine serum albumin (BSA) (New England Biolabs): Protein obtained from bovine serum. It's used as stabilizer, tampon and blocking agent.

-Ampicillin (Amp) (Sigma): β-lactam antibiotic that inhibits *E. coli* cellular division by preventing the synthesis of the cell wall. It's used for the selection of plasmid carrying cells.

-Antibody anti-cMyc (9E10):sc40 (Santa Cruz Biotechnology): Mouse monoclonal antibody that recognises the epitope located between the aminoacids 408-439 of human cMyc. Isotype IgG.

-Antibody anti-Rpb3 (Abcam): Mouse monoclonal antibody that recognises RNA polymerase II subunit Rpb3 of *S. cerevisiae*.

-Antibody anti HA (Roche): Rat monoclonal antibody that recognises the epitope HA.

-Antibody anti Ser2-P CTD (Abcam, ab5095): Rabbit polyclonal antibody that recognises the phosphorylated serine in the position 2 of the C-terminal domain repeat YSPTSPS.

-Benzamidine (Sigma): Competitive inhibitor of trypsin, trypsin-like enzymes and serine-proteases.

-Complete protease inhibitor cocktail (Roche): Mixture of protease inhibitors.

-Sodium deoxycholate 99% (Sigma): Anionic detergent used in cellular lysis buffers.

-Dietil-pyrocarbonate (DEPC) (Sigma): RNAse inactivator.

-DL-Ditioteitrol (DTT) (Sigma): Reductor agent.

-Dynabeads ® Pan Mouse IgG (Dynal Biotech): Polystyrene magnetic beads bound to human antibodies that recognise mouse IgG.

-Dynabeads ® Pan Mouse Protein A (Dynal Biotech): Polystyrene magnetic beads bound to recombinant protein A.

-Formaldehyde 37% (Sigma F-1635): Compound used to induce cross linking reactions between proteins and between DNA and proteins.

-G418, Geneticin ® (USB): Aminoglycoside antibiotic against yeast used to select yeast cells that carry the kanamycin resistance gene (KanMX).

-Glucogen (Roche 10377420). Compound is used to facilitate the precipitation of nucleic acids present at low concentrations.

-PMSF (phenylmethanesulfonyl fluoride) (Sigma): Inhibitor of serine-proteases (trypsin and quimotrypsine) and cysteine-proteases.

-Proteinase K (Roche): A serine protease with no pronounced cleavage specificity.

-Ribonuclease A (Sigma): Endoribonuclease that cleaves single stranded RNA after pyrimidine nucleotides.

-Thermostable DNA polymerase (Expand TM High Fidelity PCR System/Roche): Taq polymerase (*Thermus aquaticus)* and Pwo polymerase (*Pyrococcus woesei*) mix used in polymerization chain reactions or PCRs. Optimal elongation temperature: 68°C.

-Thermostable DNA polymerase (MBL TAq polymerase): Taq polymerase (*Thermus aquaticus)* used in polymerization chain reactions or PCRs. Optimal elongation temperature: 72°C.

-Sequenase ™ Version 2.0 DNA Polymerase (USB): Enzyme used in DNA amplification PCR reactions.

-SYBR ® GREEN: SYBR ® Premix Ex Taq ™ (Takara). Fluorescent dye for DNA detection in qPCR.

-Zymoliase 20T (USB) Mix of enzymes of *Arthobacter luteus* used to digest the cell wall of *S. cerevisiae* (20 U/mg).

-Zymoliase 100T (USB) Mix of enzymes of *Arthobacter luteus* used to digest the cell wall of *S. cerevisiae* (100 U/mg).

6.3 Strains and plasmids

6.3.1 Strains of *E. coli*

-DH5α: F-endA1 gyrA96 hsdR17 DlacU169(f80lacZDM15) recA1 relA1 supE44 thi-1 (Hanahan, 1983)

6.3.2 Strains of *Saccharomyces cerevisiae*

Table 1. Strains used in this thesis.

Strain	Genotype	Reference
BY4741	MAT a his3Δ1 leu2Δ0 met15 Δ0 ura3 Δ0	EUROSCARF
BY4742	MAT α his3Δ1 leu2Δ0 lys2Δ0 ura3 Δ0	EUROSCARF
Y05312	BY4741 ylr403w(sfp1)::kanMX4	EUROSCARF
Y15312	BY4742 ylr403w(sfp1)::kanMX4	EUROSCARF
MMY9.1	BY4742 ygl043w(dst1)::kanMX4	
MMY9.2	BY4741 ygl043w(dst1)::kanMX4	
FGY43.1B	BY4741 ygl043w(dst1)::kanMX4 ylr403w(sfp1)::kanMX4	
Y07202	BY4741 ydr007(trp1)::kanMX4	EUROSCARF
Y17202	BY4742 ydr007(trp1)::kanMX4	EUROSCARF
LMY3.1	Y07202 YJL140w(RPB4)::MYC18-TRP1	This thesis
LMY5.1	Y07202 TRP1::pADH1::3HA::DST1	This thesis
LMY7.1	Y07202 YJL140w(RPB4)::Myc18-TRP1 ylr403w(sfp1)::kanMX4	This thesis
LMY15.1	Y07202 TRP1::pADH1::3HA::DST1 ylr403w(sfp1)::kanMX4	This thesis

LMY17	trp1Δ::HisG URA3::pGAL YLR454w YJL140w(RPB4)::Myc18-TRP1	This thesis
LMY18	trp1Δ::HisG URA3::pGAL YLR454w YJL140w(RPB4)::Myc18-TRP1 ylr403w(sfp1)::kanMX4	This thesis
GYLR-3A	BY4741 trp1Δ::HisG URA3::pGAL YLR454w	Paco Malagón
GYLR-3B	BY4742 trp1Δ::HisG URA3::pGAL YLR454w	Paco Malagón
TMS5.5d	Mat α his3Δ leu2Δ0 ura3Δ0 ade2-801 lys2-801 LYS::TETR-GFP nup49Δ::HPH-MX6 (pASZ11-NUPNOP)	Olivier Gadal (Berger et al, 2008)
TMS1-1a	Mat a his3Δ leu2Δ0 ura3Δ0 ade2-801 lys2-801 LYS::TETR-GFP nup49Δ::HPH-MX6 (pASZ11-NUPNOP)	Olivier Gadal (Berger et al, 2008)
LMY6.1	Mat α his3Δ leu2Δ0 ura3Δ0 ade2-801 lys2-801 LYS::TETR-GFP nup49Δ::HPH-MX6 ygl043w(dst1)::kanMX4 (pASZ11-NUPNOP)	This thesis
LMY6.2	Mat a his3Δ leu2Δ0 ura3Δ0 ade2-801 lys2-801 LYS::TETR-GFP nup49Δ::HPH-MX6 ygl043w(dst1)::kanMX4	This thesis
LMY20-1	Mat α his3Δ leu2Δ0 ura3Δ0 ade2-801 lys2-801 LYS::TETR-GFP nup49Δ::HPH-MX6 RPL25::URA3 (pASZ11-NUPNOP)	This thesis
LMY21-1	Mat α his3Δ leu2Δ0 ura3Δ0 ade2-801 lys2-801 LYS::TETR-GFP nup49Δ::HPH-MX6 RPL25::URA3 ygl043w(dst1)::kanMX4 (pASZ11-NUPNOP)	This thesis
LMY23-1	Mat a his3Δ leu2Δ0 ura3Δ0 ade2-801 lys2-801 LYS::TETR-GFP nup49Δ::HPH-MX6 ARG3::HIS3 (pASZ11-NUPNOP)	This thesis
LMY27-1	Mat a his3Δ leu2Δ0 ura3Δ0 ade2-801 lys2-801 LYS::TETR-GFP nup49Δ::HPH-MX6 ARG3::URA3	This thesis

	ygl043w(dst1)::kanMX4 (pASZ11-NUPNOP)	
YCSG2.2a	Mat a his3Δ leu2Δ0 ura3Δ0 ade2-801 lys2-801 LYS::TETR-GFP nup49Δ::HPH-MX6 RPS3::HIS3 ygl043w(dst1)::kanMX4 (pASZ11-NUPNOP)	Olivier Gadal
YCSG2.1c	Mat a his3Δ leu2Δ0 ura3Δ0 ade2-801 lys2-801 LYS::TETR-GFP nup49Δ::HPH-MX6 RPS3::HIS3 (pASZ11-NUPNOP)	Olivier Gadal

6.3.3 Genetic analysis in yeast

Parental strains were crossed on YPD plates and incubated at 30°C. Diploids were isolated by selection in the corresponding selective medium or by micromanipulation of zygots. Diploids were then sporulated in SPO medium for 5-6 days at 26°C. The tetrads were treated with zymolyase 20T for 90 seconds at room temperature. The spores were dissected on YPD plates using a SINGER MSM system 200 micromanipulator. YPD plates were incubated at 30°C and the genotype of the spores was determined by replication in selective media.

6.3.4 Genetic replacement in yeast

The original strains were transformed with lineal DNA with terminal regions homologous to the gene to be replaced (Orr-Weaver et al, 1981) and the desired phenotype was selected. When applying the method SFH (Short Flanking Homology) we proceed as in (Wach et al, 1994).

6.3.5 Plasmids

All the plasmids that were used in this thesis are listed on table 2.

Table 2. Plasmids used in this thesis.

Plasmid	Description	Reference
Ycplac 33	CEN, *URA3*	(Gietz and Sugino, 1988)
pSK-URA3-M13		Olivier Gadal (Berger et al, 2008)

pCR4-HIS3-M13		Olivier Gadal (Berger et al, 2008)
pASZ11-NUPNOP	GFP-NUP49, mCherry-NOP1	Olivier Gadal (Berger et al, 2008)
pTetO-NAT-ura3Δ		Olivier Gadal (Berger et al, 2008)
pTetO-NAT-his3Δ		Olivier Gadal (Berger et al, 2008)

6.3.6 Transformations

6.3.6.2 Bacterial transformation

In order to transform the cells, 100 µl of competent cells were mixed with 50-100ng of DNA and the mixture was incubated on ice for 15 minutes. Cells were subsequently incubated at 42°C heat shock for 90 seconds. Then the cells were incubated for 2 minutes on ice. 1ml of LB medium was added and the cells were incubated at 37°C for 60 minutes. The transformation was plated on LB+Amp medium.

6.3.6.3 Yeast transformation

Yeast transformation was performed following the method described by (Ito et al, 1983) and modified by (Gietz et al, 1995). 50 ml of cells growing exponentially at 0.4-0.6 O.D. $_{600nm}$ were harvested by centrifugation and washed with water. The cells were resuspended in a 100 m lithium acetate solution and mixed with a solution containing the DNA, PEG4000 50%, lithium acetate 100mM, and denatured salmon sperm DNA. After incubating for 30 minutes at 30°C, the cells were subjected to a heat shock of 42°C for 15 minutes. The cells were washed with water and plated on selective medium. Alternatively, the cells were grown in YPD for 2 replicative cycles before plating on antibiotic-containing media.

6.4 Polymerase chain reaction

6.4.1 Conventional PCR

This method allows the exponential amplification of DNA fragments by a thermostable polymerase and two oligonucleotides that are required as primers of the enzymatic reaction (Saiki et al, 1985). The reaction is prepared by mixing in a reaction tube 42 µl of water, 5 µl of 10X polymerase buffer, 1 µl dNTPs mix (10mM each), 1 µl primers mix (0.1 nmoles/µl each), 1

µl template DNA and 0.4 µl Expand TM High Fidelity polymerase or 0.7 µl Taq polymerase. The reactions were incubated in a Biorad T100 thermocycler. The program used was: 1 cycle of 2 minutes at 95°C; 35 cycles consisting of a) 30 seconds at 95°C, b) 30 seconds at the specific annealing temperature of the primers used, c) 1min/kb of PCR product at 72°C in case of the Expand TM polymerase or 68°C for the MBL Taq polymerase; and 1 final cycle of 10 minutes at 72 or 68°C, depending on the polymerase used for the reaction.

6.4.2 Quantitative PCR

Quantitative PCRs (Q-PCR) allow the determination of the amount in which a determined fragment of DNA is present in a sample. It combines the amplification of said fragment of DNA by PCR with the measure of the quantity of double stranded DNA by the detection of the fluorescence emitted by SYBR ® Green, a DNA-binding agent. The reaction mix (4 µl DNA solution, 0.5 µl primers mix, 0.5 µl water and 5 µl SYBR ® Green) is maintained at cold temperature and run in 384 well plates in a Light Cycler 480 II thermocycler (Roche). Manufacturer instructions were followed.

6.4.3 Oligonucleotides

The names and sequences of the oligonucleotides used in this thesis are detailed in the table 3. All the primers were designed in the software Oligo 4.01 or PrimerExpress 2.0 in the case of those primers that were to be used in q-PCR.

Table 3. Oligonucleotides used in this thesis.

Oligonucleotide	Sequence	Use
Intergenic Chr:V F	TGTTCCTTTAAGAGGTGATGGTGAT	qPCR
Intergenic Chr:V R	GTGCGCAGTACTTGTGAAAAC	qPCR
ADH1 1 up	GCACGGTGACTGGCCATT	qPCR
ADH1 1 low	TCTTCCAGCCCTTAACGTTTTC	qPCR
ADH1 2 up	TGCTAACTTGATGGCCGGTC	qPCR
ADH1 2 low	AGACTCTGTAACCCATAGCCTTGG	qPCR
ADH1 3 up	ACGTCGGTAACAGAGCTGACAC	qPCR
ADH1 3 low	TTCTGGCAAGGTAGACAAGCC	qPCR
PHO88 1 up	CATGTTGGTCATGATGCAACTCT	qPCR

PHO88 1 low	GTTGGGTCCTCCATGTCAATG	qPCR
PHO88 2 up	GGTAATGCTATGTCCGGCGA	qPCR
PHO88 2 low	TCTGACGGTAGTAACTTGCAGCTT	qPCR
PHO88 3 up	CCTCTTCGGTAAGCCTGCAA	qPCR
PHO88 3low	GGAGCCTTGAATGGTCTCTTCA	qPCR
HXT1 1 up	GGCCATGAATACTCCAGAAGGT	qPCR
HXT1 1 low	GAAACCACCGAAAGCAACCA	qPCR
HXT1 2 up	CGTTATTTGGTTGAAGCTGGC	qPCR
HXT1 2 low	AAGATGCAGTACCAGCGGCT	qPCR
HXT1 3 up	TCATGGGCTGTATGGTTTTCG	qPCR
HXT1 3 low	GCGCCTCTCTTGGATACTGG	qPCR
HXK2 1 up	TGCCAAAGGAATTGATGCAA	qPCR
HXK2 1 low	CAATTCGGAAATGAAGTGCTTG	qPCR
HXK2 2 up	GGCCATCAACTGTGAATACGG	qPCR
HXK2 2 low	GGCCTGGTCTTGGAGATTCTTC	qPCR
HXK2 3 up	GTCCGTTTGTGGTATTGCTGC	qPCR
HXK2 3 low	TGAGGTTTGAGTCCAGCCGTA	qPCR
RPS3 1 up	TTTTTGAACATTGTTTTGATAACTGAAA	qPCR
RPS3 1 low	AGAGATTAAAGCGACCATTTTTGTAGT	qPCR
RPS3 2 up	AAGAGATTCAAGTACGCTCCAGGT	qPCR
RPS3 2 low	GCGGACAAACCACGGTCT	qPCR
RPS3 3 up	GGTTGTGAAGTTGTTGTTTCCG	qPCR
RPS3 3 low	AAACCGTCAGCAAATTTCATAGC	qPCR
RPS8 1 up	CGTCACAAAAGATCCGCTACC	qPCR
RPS8 1 low	TCTTGGTGTTGGCTGGTTGA	qPCR
RPS8 2 up	CCAAGAAGACCAGAATTGCTGG	qPCR
RPS8 2 low	AGCATCAATTTGGACAATGGC	qPCR
RPS8 3 up	GCTGCTTCTGCCAAGATCG	qPCR
RPS8 3 low	CGGATTGACCTGGTCTGGAA	qPCR
RPL5 1 up	CCAAAAAGACGCTAAGTCCTCTG	qPCR
RPL5 1 low	CTTGGCCTTGTGTTGGGTG	qPCR
RPL5 2 up	TGGGTTTGGACGAAACTTACAA	qPCR

RPL5 2 low	AGAGCACCGAAAACTCTGGC	qPCR
RPL5 3 up	CACTTCTGCTCACGAAGCTATCA	qPCR
RPL5 3 low	GATCTTGGCAGCAACACGAG	qPCR
RPL25 1 up	CACGAGAAAATTGAGAGGAAGATAGA	qPCR
RPL25 1 low	CGTGTGGTTTTCGTATCTCATCA	qPCR
RPL25 2 up	AGCAACGTAATTATCGGGCTCA	qPCR
RPL25 2 low	AACTTCCACAGCATCAACGCT	qPCR
RPL25 3 up	TGGTTTTCCAAGTTTCCATGAA	qPCR
RPL25 3 low	AACTTCGTATAATTCCTTGACGGC	qPCR
NTS2 up	AGTGAGGAACTGGGTTACCCG	qPCR
NTS2 low	TTTCTTTTGCCCTCTCTGTCG	qPCR
rDNA1 up	AATTGAAGTTTTTCTCGGCGA	qPCR
rDNA1 low	ATGAAGTACCTCCCAACTACTTTTCC	qPCR
rDNA2 up	AACAGTCTCATCGTGGGCA	qPCR
rDNA2 low	TGAGAGGAGGTTACACTTGAAGAAT	qPCR
rDNA3 up	CAATAGCGTATATTAAAGTTGTTGCAGTT	qPCR
rDNA3 low	AAAGTCCTGGTTCGCCAAGAG	qPCR
rDNA4 up	AACATTCTGTTTGGTAGTGAG	qPCR
rDNA4 low	AGTATAAAAAAAGATTAGCCG	qPCR
rDNA5 up	TAACAGCTTATCACCCCGGAA	qPCR
rDNA5 low	CGGTTATCAGTACGACCTGGC	qPCR
ARG3 M13 up fw	GATTATTTTGCAATGTCAATTAAAAAAAAAAAATGTTAGTAAAACTATGTTACATTCCAAGCAAATAAAGCACTTGGTTAAACGTAAAACGACGGCCAGT	PCR
ARG3 M13 low rev	GACAACTCGAAGGAATTTGTTGGCAGTGTATATATTTAAATTTTTAGACCGCGGTCTGGCTGTCTTAAAAACGTTAATTTCCAGGAAACAGCTATGACCATG	PCR
ARG3 check up fw	GTCTCTGTGGCTGATCCAAAC	PCR
ARG3 check low rev	GGTCATGTCATGTCGTCCCG	PCR
DST1 out up	TCTGCCTATTCATTTTATCG	PCR
DST1 out low	CTTAGCATTATGTGTGTGGCTG	PCR
ADH1 up	TCCAAAGCCAAAGGCCAACGA	Run on

ADH1 lo	TTGGCACCAGCTGGCATACCG	Run on
RPS3 up	AAGAGATTCAAGTACGCTCCAGGT	Run on
RPS3 lo	AAACCGTCAGCAAATTTCATAGC	Run on
RPS8 up	ATTCTCGTCACAAAGATCC	Run on
RPS8 lo	GTCTGGAAGAGATACAAGCG	Run on
RPL5 up	ATGGCTTTCCAAAAAGACGC	Run on
RPL5 lo	GATCTTGGCAGCAACACGAG	Run on
RPL25 up	GCTACTGCCGCTAAGAAAGC	Run on
RPL25 lo	TAGCAATGTCCAAAGCATCG	Run on
PHO88 up	TGGTCATGATGCAACTCTCC	Run on
PHO88 lo	TACCGGCTCTTTCAGCTTCT	Run on
HXK2 up	TGCCAAAGGAATTGATGCAA	Run on
HXK2 lo	TGAGGTTTGAGTCCAGCCGTA	Run on
HXT1 up	GGCCATGAATACTCCAGAAGGT	Run on
HXT1 lo	GCGCCTCTCTTGGATACTGG	Run on
Rpb4 Myc up	TTGGAAAGGATACTAAAGGAATTGTCAAACCTAGAAAC ACTCTAT	PCR
Rpb4 Myc low	CTGTTAAATAGGCCTAACTTCCACAGGGTGAAAATTCAT TACTAG	PCR
Rpb4 Myc test up	TAGAGACCAAGAAACCGTCG	PCR
Rpb4 Myc test low	AGCATCTTACGCTGTTAGGC	PCR
Sfp1 test up	GGCTGTACCCACACCGAGTG	PCR
Sfp1 test low	CGCAAATCGTTTTCATGACG	PCR
oJW102	GCGGTGACCCGGGAGATCTGAATT	DNA amplification
oJW103	GAATTCAGATC	DNA amplification
Primer A	GTTTCCCAGTCACGGNNNNNNNNN	DNA amplification (Affymetrix)
Primer B	GTTTCCCAGTCACGGTC	DNA amplification (Affymetrix)

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6.5 *In vivo* labeling of nascent RNA by elongating RNA polymerase II (transcriptional run-on)

Run on assays were performed as previously described (Bentley, 1995; García Martínez et al, 2004) with minor modifications. The run-on is a technique in which the nascent mRNA is radioactively labelled by the incorporation of radioactive nucleotides in its sequence during transcription.

25 ml of yeast culture were incubated at 30°C and collected when it reached an O.D. $_{600nm}$ of approximately 0.5. For those cultures to be treated with 6AU, a concentration of 200 μg/ml was added to the culture in the same volume of as the culture prior to harvesting at the designated times of treatment.

The cultures were then harvested by centrifugation at 3000 rpm for 3 minutes and then washed in 5 ml of 0.5% sarkosyl solution. The supernatant was discarded after another centrifugation of 3 minutes at 3000 rpm. The pellet was then resuspended in 60 μl of double-distilled water. Then it was added 80 μl of a mix consisting of: 60 μl 2.5x transcription buffer (50 mM Tris/HCl pH 7.7; 500mM KCl; 80 mM MgCl$_2$), 8 μl of ribonucleotide mix (ATP, GTP and CTP) at a concentration of 10 mM, 3 μl 0.1M DTT and 10 μl [α-32P]UTP (3000 Ci/mmol), resulting a final volume of 150 μl approximately. The mix was incubated at 30°C for 5 minutes to allow transcriptional elongation and the reaction was stopped by the addition of 1 ml of cold double-distilled water. After centrifugation, the supernatant was discarded in order to eliminate non-incorporated nucleotides from the mix and total RNA was extracted following the acid phenol protocol. The RNA was precipitated overnight in a solution of 2.5 volumes of 96% ethanol and 0.1 volumes of 5M lithium chloride. Once precipitated the RNA was washed with 70% ethanol and dried before resuspending in 100 μl water.

Radio-labelled RNA from the run-on was fragmented and denatured prior to hybridisation by adding 25 μl of 0.2 N NaOH and incubating on ice for 5 minutes followed by the addition of 25 μl of 0.2 N HCl with the object of neutralise the reaction.

Slot blotted membranes were performed as formerly described (Rodríguez-Gil et al, 2010). A Hybond N+ membrane (Amersham) was placed into a PR600 Slot blot device (Hoefer Scientific Instruments). Each well of the device was loaded with 500 ng of dsDNA probes diluted in 0.5 ml 0.4M NaOH 200 mM EDTA, previously boiled to denature the DNA. The solution was forced through the membrane by applying a vacuum pump to the slot device. The membrane was then washed by rinsing it in 2X SCC (0.6M NaCl, 60mM sodium-citrate).

Double-strand immobilised probes were obtained by PCR using the primers listed before. Membranes were exposed in Fuji BAS screens and developed with a FUJIX FLA5100 device. Signals were quantified using ImageGauge.

6.6 Chromatin immunoprecipitation and DNA amplification for RPCC and ChIP on chip

The ChIP assay was performed as previously described (Rodriguez-Gil et al, 2010).

6.6.1 Sample preparation

Yeast cells were inoculated in 50 ml of liquid medium and were incubated at 30°C until they reached an O.D. $_{600nm}$ of 0.6 in exponential growth. In those experiments that required a treatment with 6AU the drug was added at this point at a final concentration of 100 μg/ml. For the sample corresponding to time 0 minutes, the same volume of medium without the drug was added so the experimental conditions would be similar for all the samples analysed. The cross-link of DNA and proteins was induced by the addition of formaldehyde to a final concentration of 1% followed by 10 minutes of incubation at room temperature. In order to stop the crosslinking reaction 2.5 M glycine was added to a final concentration of 2% and the samples were incubated at room temperature for 5 minutes while mixing occasionally. After this, the cells were washed 3 times with cold TBS buffer (20 nM Tris-HCl pH 7.5, 0.15 M NaCl) to eliminate the formaldehyde. The samples were kept at -20°C until use. Concomitantly to the sample preparation, 50 μl per sample of magnetic beads (Dynabeads) were washed twice with 1 ml PBS/BSA buffer and incubated with 50 μl of an antibody solution (the concentration varies depending on the antibody) at 4°C in low agitation overnight.

6.6.2 Chromatin immunoprecipitation (ChIP)

The samples were thawed on ice and resuspended in 300 μl of lysis buffer. For the cell breakage, the samples were transferred to another tube with 300 μl of glassbeads and homogenised with the help of a FastPrep (3 cycles of 30 seconds at 5 m/s). The homogenized sample was collected in another tube and sonicated in a Bioruptor (Diagenode) in order to fragment the DNA (2 periods of 15 minutes at maximum level with 30 seconds on/off cycles). The sonication resulted on DNA fragments of approximately 400 base pairs in size. The supernatant was recovered after centrifugation and 10 μl were transferred to another tube that was kept on ice to be used as control of the immunoprecipitation (whole cell extract) in the quantification of the experiment. The mix of antibodies+ dynabeads previously prepared was washed with 600μl of PBS/BSA four times in order to eliminate the excess of antibody and was resuspended in 30μl of PBS/BSA for each sample. The magnetics beads were added to the

supernantant and incubated for 90 minutes at 4°C on rotation. Once the incubation was completed the samples were washed: twice with 1 ml of lysis buffer; twice with 1 ml of buffer lysis/360 mM NaCl; twice with 1 ml of washing buffer and finally once with 1ml TE. The samples were then eluted with 50µl of elution buffer by incubating the sample at 65°C. 60µl were transferred to a new tube and incubated 12-14 hours at 65°C in order to revert the cross-linking. The sample corresponding to the raw extract received the same treatment, as well as all the following procedures applied to the immunoprecipitated samples. Once the cross-linking was reverted, the samples were treated for 90 minutes with proteinase K (0.5µg/µl) and the DNA was purified using phenol:chlorophorm (1:1) and chlorophorm:isoamylic (24:1) . DNA was precipitated in cold isopropanol (2.5 volumes) with the help of glycogen (0.5µg/µl) and NaCl (0.5M). After DNA precipitation, the samples were resuspended in double-distilled water, aliquoted in 10µl samples and kept at -20°C until use. DNAs were analysed by real-time quantitative PCR.

Solutions for ChIP

PBS/BSA	HEPES 250mM pH 7.5 (KOH)	PMSF 0.1M
125mg/ml PBS (1X)	11.915g in 200ml H_2O Adjust pH with KOH	0.43g in 25ml of isopropanol

Lysis Buffer (25ml):	
HEPES	5ml
NaCl 5M	700µl
EDTA 0.5M pH8	50µl
Triton X-100	250µl
Nadeoxycholol	0.025g
PMSF	250µl
Cocktail	500µl
Benzamidin	0.038g
H_2O	Until 25ml
Add 1.8ml NaCl for Lysis Buffer NaCl	

Washing Buffer		Elution Buffer (50ml)	

(25ml)			
TRIS	250µl	Tris 1M pH8 (HCl)	2.5ml
LiCl	1.25ml	EDTA 0.5M	1ml
NP-40 (IGPAL)	125µl	SDS 10%	5ml
Nadeoxycholol	125mg	H_2O	41.5 ml
EDTA	50µl		
H_2O	Until 25 ml		

6.6.3 Quantification of ChIP by qPCR

The relative enrichment of a protein in a given DNA sequence was obtained by dividing the signal from the immunoprecipitate (determined by the quantitative PCR program) by the signal obtained for the whole cell extract. In many of these essays a sequence from the intergenic region of chromosome V was used as a control to determine the specific recruitment of a protein to a given DNA sequence. Apart from this control, we also analysed a sample not incubated with the antibody as a negative control.

6.6.4 Amplification of DNA by ligation-mediated PCR

The protocol of ligation mediated PCR was performed like described in (Pelechano et al, 2009) (García-Martínez et al, 2011), that is a modification of the original protocol described in Richard Young's laboratory. DNA was blunted by T4 phage DNA polymerase in a reaction volume of 124 µl (T4 DNA polymerase buffer, 40 µg/µl BSA, 80µlM dNTPs, 0.6U T4 DNA polymerase). The reaction was incubated for 20 minutes at 12°C. After phenol:chloroform:isoamylic alcohol extraction, DNA was ethanol precipitated and ligated in a final volume of 50 µl with the annealed linkers oJW102 and oJW103 (1.5µM of each primer). The reaction was carried out overnight at 16°C and ligated DNA was precipitated and resuspended in 25 µl milliQ water. The ligated DNA was dissolved in a final volume of 40 µl (1X DNA polymerase buffer, 2mM $MgCl_2$, 0.25 mM dNTPs, 1.25 µM oligonucleotide oJW102). The reaction was started by incubating for 2 minutes at 55°C, then pausing to add 10 µl of the reaction mix (1X DNA polymerase buffer, 2mM $MgCl_2$, and 5U of DNA polymerase). The program was resumed for 5 minutes at 72°C, 2 minutes at 95°C and 33 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 2 minutes at 72°C. DNA was precipitated overnight. Next, this

amplified DNA is used as a template for a single cycle of PCR using a cytosine labelled with 33-phosphorous (dCTP[α-33P]). This DNA is purified and hybridized in a nylon membrane.

6.7 Radioactive labeling of nucleic acids

6.7.1 Radioactive labeling of genomic DNA and DNA probes (random priming)

Genomic DNA and DNA probes were labelled with [α-32P]dCTP using a basic protocol for radioactive probes. In this case, 50-200 ng of DNA were resuspended in 35 µl of double-distilled water and boiled in order to denature the DNA. Afterwards, 5 µl of a 10X solution of hexanucleotides (Roche), 5 µl of a mix of dATP, dGTP, and dTTP 0.5 mM, 20 µCi of [α-32P]dCTP, and 2U Klenow DNA polymerase were added. The reaction was incubated at 37°C for 1 hour in a final volume of 50 µl. The nucleotides that were not incorporated in the reaction were eliminated through a Sephadex G-50 column. The DNA used for the labelling of probes was previously obtained by conventional PCR using the oligonucleotides listed in table (x).

6.8 DNA arrays

RNA polymerase II immunoprecipitation was carried out using an anti-Rpb3 antibody. After crosslinking reversal, the fragments of enriched DNA that were obtained were unspecifically amplified and labelled following Affymetrix Chromatin Immunoprecipitation Assay Protocol P/N 702238 with minor modifications. Genomic DNA controls were processed in parallel. After PCR amplification with dUTP, the samples were purified using Qiagen QIAquick PCR purification kit. DNA quality and quantity were checked using a NanoDrop ND-1000 spectrophotometer. Afterwards, 0.5 µg from each sample were used to hybridise the GeneChip *S.Cerevisiae* Tiling 1.0R custom arrays. This step was carried out in the Multigenic Analysis Service of the University of Valencia. The resulting CEL files were normalised and the intensities of the signals were extracted using the TAS (Tiling Analysis Software) developed by Affymetrix specifically for the analysis of tiling array chips. The samples were subjected to quantile normalization. The resulting text files were read using R-language scripts and the Bioconductor platform. We determined the average intensity values of Rpb3 IP for each open reading frame (ORF) and also the distribution of IP signal along the length of each gene.

6.8.1 Macroarray production

The DNA macroarrays were produced by the Section of DNA Chips-S.C.S.I.E of the University of Valencia following the protocol described by (Alberola et al, 2004). Double stranded DNA probes were deposited over positively charged nylon membranes (Amersham

HybondTM N+) using a chip production robot BioGridTM (Biorobotics). The probes were impressed directly on the membrane without PCR purification. The membranes were kept moist during all the fabrication process, by placing them over three sheets of Hybond blotting paper soaked in denaturing solution (1.5M NaCl, 0.5M NaOH). For the impression, needles of 0.4 mm of diameter were used. This needles deposit 20 nl every time they touch the membrane. The PCR products were deposited 5 times, in such a way that every dot contained between 20 and 30 ng of DNA in a diameter of 0.6 mm. After the impression, the membrane was neutralised immersing it in a solution (1.5M NaCl, 0.5M Tris-HCl pH 7.2, 1mM EDTA pH 8.0) for 1 minute. Finally the membranes were dried on filter paper.

6.8.2 Macroarray hybridisation

Chip membranes were pre-hybridised for 1 hour in a buffered solution (0.5M phosphate buffer, 0.1mM EDTA, SDS7%, pH 7.2) at 65°C and subsequently all the labelled DNA or RNA was added (3.5×10^7 dpm approximately) in 3.5 ml of the same solution. It was incubated for 36-40 hours in a hybridization oven at 65°C. After hybridisation the membrane was washed twice with 1XSSC and SDS 10% for 10 minutes and twice with 0.5X SSC, 0.1% SDS for 20 minutes. The filters were exposed for 5 to 7 days in Fuji BAS screens and the quantity of labelled RBA bound to the filter was quantified with a β radiation analyser FUJIX FLA 3000.

6.8.3 Macroarray deshybridisation

After every hybridisation and its subsequent exposition macroarrays were washed with a solution containing sodium phosphate pH 7.5 and 0.1% SDS in order to eliminate the radioactivity from the filters. These washes were done in three consecutive steps in which the solution was added boiling to the membranes and kept in agitation for 10 minutes.

6.8.4 Macroarray quantification

Once hybridised and washed, the macroarrays were exposed in a FUJI cassette with a Fuji BAS screen for 5-7 days. These screens were read with a FUJIX FLA3000 scanner, using the software Fujifilm Image Reader 2.01. The images were analysed with the software Array Vision (Imaging Research Inc).

6.9 Normalisation and data processing for run-on and RNA polymerase II ChIP on chip (RPCC)

All the images for chip hybridisations were obtained using a FLA 3000 phosphorimager (Fujifilm) of 16 bits of depth and a resolution of 50 μm, The images were analysed with the help of the program ArrayVision (Imaging Research) excluding all the dots in which the signal

was not at least 1.35 times stronger than the background. To each of those values the Artifact Removed Median (sARM) was substracted, being this number the value of the median of the pixels for each point, after eliminating all the possible artifactual stains and substracted the intensity of the background.

All the samples were done for triplicate, shuffling the membranes between samples in order to avoid a possible bias. The analysis of the replicates was done using the software ArrayStat (Imaging Research) with a proportional model at logarithmic scale. The data was normalised along replicates assuming independent conditions and a minimum of 2 valid replicates (similar values) for each gen. In order to calculate the error associated to each measure an estimation of the error was used to adjust the curve. Finally anomalous values were eliminated.

6.9.1 Run on and RPCC signal normalisation

To perform a GRO assay, the mRNA that proceeds from a run-on experiment is hybridised in a nylon membrane that contains all the ORFs described for *S. cerevisiae*.

In order to avoid bias in the run-on signal due to the membrane, it was hybridised with radioactively labelled genomic DNA (gDNA) for each chip membrane and the run on value for each sample was divided by the corresponding gDNA signal. Due to the fact that run on incorporates UTP to a single RNA strand, while DNA incorporates dCTP to both strands the values were corrected taking into account the proportion of nucleotides for each probe.

The data obtained in ArrayStat were represented after normalisation by median and standard deviation (z-score log2) to compare the tendency of different genes inside a same set, as well as to allow the comparison between run on and RPCC samples because the unspecific amplification that RPCC samples suffer prevent the use of those data as absolute levels of Rpb3 present of the genes.

To calculate the enrichment in total polymerases (RPCC) the values for each immunoprecipitated sample were divided between the values obtained for the whole cell extract and they were compared similarly to the run on using the software ArrayStat. To delimitate the minimal background signal in these experiments the 1000 genes with less RPCC signal were eliminated. Aditionally, the background signal was substracted from the remaining samples. The background signal is the average for 164 genes that were among the 1000 genes with the less signal in each condition. Once obtained a value for each sample, they were

normalised with respect to their average and their standard deviation as it was done for run on samples.

6.10 Tiling array ChIP on chip experiment and data processing

For Rpb3 and Rpb4-Myc ChIP on chip experiments, chromatin immunoprecipitation was performed as above and, after crosslinking reversal, the obtained fragments (300 bp approximately) of enriched DNA were amplified unspecifically and labeled following Affymetrix Chromatin Immunoprecipitation Assay Protocol P/N 702238. Genomic DNA controls were processed in parallel. 10 μl of each sample were amplified using Sequenase ™. The reaction mix consisted of 10 μl purified DNA, 4μl 5X Sequenase ™ reaction buffer and 4μl Primer A (200μM) for each reaction. The cycle conditions for random priming were 95°C for 4 minutes, snap cool on ice and hold at 10°C. Next, 2.6μl of "first cocktail" (0.1μl 20mg mg/ml BSA), 1μl 0.1M DTT, 0.5μl 25 mM dNTPs and 1μl diluted Sequenase ™ 1/10 from 13U/μl stock) were added to each reaction and put back in the thermocycle for the following program: 10°C for 5 minutes, ramp from 10°C to 37°C over 9 minutes, 37°C for 8 minutes, 95°C for 4 minutes, snap cool on ice and 10°C hold. Then another 1μl of cocktail was added to each sample and these steps were repeated for two more cycles. The samples were kept at a 4°C hold. After PCR amplification with dUTP, the samples were purified using Qiagen QIAquick PCR Purification Kit (50) (Cat.No. 28104). About 56μl of first round purified DNA were collected for each reaction. The amplification PCR was performed as usual but using 20μl of first-round DNA from the previous step, 3.75μl of a 10mM dNTPs + dUTP mix and 4μl of 100μM Primer B. The cycling conditions were: 15 cycles consisting of 95°C for 30 seconds, 45°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, and 15 cycles of 95°C for 30 seconds, 45°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, adding 5 seconds for every subsequent cycle. DNA quality and quantity were checked in a 1% agarose gel and using a NanoDrop ND-1000 Spectrophotometer.The samples were purified using the QIAquick PCR purification kit (Qiagen). Then 0.5 μg of each were used to hybridize GeneChip S.Cerevisiae Tiling 1.0R custom arrays. This step was carried out in the Multigenic Analysis Service of the University of Valencia. The obtained CEL archives were normalized by quantile normalization and the intensities of the signal were extracted using the TAS (Tiling Analysis Software) developed by Affymetrix. The resulting text files were read using R scripts to adjudicate probe intensities to genes. The log_2 values of the median intensities of the chosen different group of genes were represented. In order to compare the data between different experiments the values were normalized by median and standard deviation (z-score log_2).

6.11 Localisation of genes inside the yeast nucleus.

Using Fluorescent Repressor operator system (FROS), TetO sequences are inserted near the gene of interest in a yeast cell expressing the nuclear pore protein Nup49 fused to GFP to visualize the nuclear envelope, and the nucleolar protein Nop1 fused to mCherry labeling the nucleolus. To analyze the spatial location of a locus in the nucleus of Saccharomyces cerevisiae, the three dimensional position of the locus relative to the nuclear envelope, the nuclear center and the nucleolus is computed from a large number of individual nuclei (typically 1000). Asynchronous live-cell populations are imaged in three dimensions (3D), each image consisting of approximately 200 cells. Then an automated module identify cell in interphase (G1 and S phase) in the fluorescence image and for each individual cell are computed the 3D coordinates of the locus, the nuclear center and the nuclear "centroid". From the computed distances, high-resolution probabilistic gene maps are generated. From this distribution, described as gene-map, the probability density of gene position is plotted relative to median nuclear envelope and nucleolus.

The labelling of the genes was done in two steps.

After choosing the genes of interest, the genes *URA3* or *HIS3* were integrated about 200 or 500 bp from the 3' or 5' end of them. In order to do so, the marker casettes were amplified from a plasmid (pSK-URA3-M13 or pCR4-HIS3-M13) using oligonucleotides that carried the M13 sequences and an extension of 50 bp homologous to the point of insertion. With this PCR product we transformed the strain TMS1-1a that carried a plasmid containing the labelled versions of NUP49 with GFP in order to visualize the nucleus and NOP1 with mCherry to visualize the nucleolus. This strain also produces TETR-GFP from a LYS2::TETR-GFP fussion, that will target the TETO repeats inserted in the gene of interest. The transformants were selected in SC-URA or SC-HIS media and the integration was checked by PCR.

The labelling of the gene was performed by transformation with an EcoRI linearized plasmid. Depending on the marker cassette used the plasmids could be pTetO-Nat-ura3Δ or pTetO-Nat-his3Δ. The integration of this plasmid disrupts the auxotroph marker which is checked by striking in SC-media. The transformants were checked individually under the microscope to determine the clones with better labelling that were used for gene map generation.

The *dst1Δ* strain was constructed by crossing the labelled strains with a *dst1Δ* mutant and the desired spores were selected by micromanipulation. The localization experiments were performed in four independent clones, to exclude strain specific effect.

The treatment of the samples was carried out as follows:

The cells were grown in 50 ml SC medium until they reached exponential growth (0.6 OD 200 aprox.) At this point the cultures were divided in two and an equal volume of tempered (30°) SC-URA or SC-URA 200µg 6AU respectively were added to the flasks. The samples for microscopy were collected after different times.

6.11.1 Fluorescence microscopy of living yeast cells

From each culture was taken 1 ml that was centrifuged in an eppendorf (3.2 rpm 2.5 minutes) and washed with the same medium. The supernatant was carefully discarded and the remaining pellet was resuspended in 5 µl of medium. In case of cultures treated with 6AU all the media used during the microscopy analysis was prepared at the same final concentration of the drug (100 µg/ml). Then these cells were quickly deposited on an 2% agarose pad on a slide and covered with a cover slip sealed with hot paraffin in order to avoid the dissecation of the sample. The imaging chamber of the microscope was maintained at 30 ° during all the acquisition process.

The images of the preparations were taken using a confocal microscope with an IX81 Olympus microscope and a Yokogawa spinning disk unit controlled by Andor revolution IQ1 software (Andor Technology) in the installations of the CNRS in Toulouse, France. (https://www-lbme.biotoul.fr/plateaux/ANDOR-DEFPUBLI/tour%20Andor%20def.html)

To obtain 3D images, z stacks of 41 images with 250 nm steps were taken. The confocal images were analysed with a nucloc (www.nucloc.org) MATLAB script (Berger et al, 2008).

6.12 Determination of the elongation rate of RNA polymerase II

The cultures were grown in galactose containing medium to allow the expression of the gene. When the cultures reached an OD600 of 0.5 25 ml of a 50% solution of glucose (2% total concentration) was added to the medium after harvesting the sample that corresponded to time 0 of the treatment.

To do so, each time was divided by the IP value that corresponded to the previous time point, in order to take into account only the polymerases that remain transcribing at each moment. Then the different time points were represented versus the length at which the IP was determined and the equation of the polynomial tendency line for that dispersion of points was calculated. Using the engine available at www.wolframalpha.com the equation was solved, obtaining the area under the curve. To determine the distance that the polymerase has progressed during elongation that value was subtracted from the length of the gene and the data represented versus the time. From this representation it was obtained another tendency line which slope indicates the velocity at which the polymerase proceeds during transcription elongation. Those set of data that presented an R inferior to 0.95 were discarded. A T student test was also performed to be sure that the obtained results were significant.

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